Docket No.: 300622000123
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Chaitan KHOSLA et al.	
Application No.: 09/925,236	
Filed: Aug. 8, 2001	Art Unit: 1652
For: RECOMBINANT PRODUCTION OF NOVEL POLYKETIDES (to be amended)	Examiner: Nashaat T. Nashed, Ph.D.

DECLARATION OF LEONARD KATZ

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Leonard Katz, declare as follows:

1. I am the same Leonard Katz listed as an inventor on U.S. patents 5,824,513 and 6,004,787. I have been working in the field of polyketide synthesis for more than 20 years. A copy of my *curriculum vitae* is attached as Exhibit A.

2. I have reviewed the specification and claims currently pending in the above-referenced case. I understand that the Office has rejected the pending claims as non-enabled for anything other than the host cell *Streptomyces coelicolor* transformed with the nucleic acid from the 6-deoxyethronolide B gene cluster. I understand that it is asserted that the specification does not

enable a person skilled in the art to make and use the invention commensurate in scope with the claims as currently pending. I further understand that it is asserted that host cells other than *S. coelicolor* would not be workable in the claimed method and that knowledge regarding nucleic acids encoding gene clusters other than that of 6-dEB would not permit one to predict that the method would be workable for these clusters.

3. From my own experience in practicing this technology, I state that the methods described in the specification of the above-referenced application have been successfully applied to a large number of modular polyketide synthase-encoding genes and have been successful in a wide range of actinomycete cells. Among the PKS encoding gene clusters that have been successfully used in the claimed method are those encoding the macrolides that are associated with the antibiotics midecamycin, narbonolysin, oleandomycin, epothilone, picromycin/methymycin, megalomycin, tylosin, spiramycin and chalcomycin, including hybrids formed from two of the last three mentioned PKS. Many of these were expressed specifically from inserts in pRM5 host vectors. Among the actinomycete host cells to which the method has been successfully employed are *Streptomyces fradiae*, *Streptomyces lividans, Streptomyces glaucescens*, and *Saccharopolyspora erythraea.*

4. Some of this work has been published. See, for example, Pfeifer, B. A., *et al., Microbiol. Molec. Biol. Rev.* (2001) 65:106-118 which lists expressed PKS for various antibiotics on page 114 and alternative actinomycetes to *S. coelicolor* on page 116; Reeves, C. D., *et al., Chem. & Biol.* (2004) 11:1465-1472 which reports synthesis of hybrid 16-membered macrolides in *S. fradiae* and Rodriguez, E., *et al., Appl. Microbiol. Biotechnol.* (2004) 66:85-91 which describes

successful synthesis of macrolides that require methoxymalonyl ACP precursors in *S. fradiae* when the host is supplied a mechanism to prepare the required methoxymalonyl ACP precursor.

5. Based on the actual practice of the methods described in the above-referenced application, I can state that it is enabled for a wide range of modular PKS and for a wide range of host cells.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Hayward (city), CA (state), on 13 (day) January (month) 2005.

(Leonard Katz)

1/13/05

LEONARD KATZ

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Nationality: USA

ADDRESS

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EDUCATION

1970 Ph.D. Molecular Genetics, University of California, Santa Barbara, CA; Thesis Advisor: Professor Ellis Englesberg; *Genetic and Physiological Studies on*
1965 B.Sc. Microbiology, McGill University, Montreal, Canada
the Regulation of the ara Operon in Escherichia coli B/r.

EXPERIENCE

2001- Vice-President, Biological Sciences, Kosan Biosciences, Inc., Hayward, CA 94545
2000 Executive Director of Biological Sciences and Senior Research Fellow, Kosan Biosciences , Hayward, CA 94545
1999 Executive Director, Biology & Project Management, Kosan Biosciences, Inc. Hayward, CA 94545
1996-1998 Research Fellow, Volwiler Society & Senior Project Leader, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL 60064: *Antibacterial Biology*
1994-1996 Research Fellow, Volwiler Society & Senior Group Leader, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL 60064: *Anti-infective Molecular Biology*
1993-1994 Associate Research Fellow, Volwiler Society & Senior Group Leader, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL 60064: *Anti-infective Molecular Biology*
1990-1993 Associate Research Fellow, Volwiler Society & Laboratory Head, Microbial Genetics Laboratory, Corporate Molecular Biology, Abbott Laboratories, Abbott Park, IL 60064: *Erythromycin Genetics, Prokaryotic Cloning & Expression*
1985-1990 Laboratory Head, Microbial Genetics Laboratory, Corporate Molecular Biology, Abbott Laboratories, Abbott Park, IL 60064: *Erythromycin Genetics*
1981-1985 Senior Scientist, Corporate Molecular Biology, Abbott Laboratories, Abbott Park, IL 60064: *Erythromycin Genetics*
1979-1980 Senior Scientist, Chemical and Agricultural Products Division, Abbott Laboratories, North Chicago, IL 60064: *Strain Development*
1977-1979 Senior Scientist, Schering-Plough Inc., Bloomfield, NJ 07003: *Antibiotic Genetics*

1974-1977 Assistant Professor, Biology Department, New York University, New York, NY 10003: *Teaching & Research*

1970-1974 Post Doctoral Research Biologist, University of California, San Diego, CA : *Plasmid Replication*

1965-1970 Research Assistant, University of California, Santa Barbara, CA: *Microbial Genetics*

AWARDS, GRANTS & SCIENTIFIC ACTIVITIES

2004 Convenor, *Symposium on Genetic Engineering for Therapeutic Improvements*, Genetics and Molecular Biology of Industrial Microorganisms/Biotechnology of Microbial Products Meeting, San Diego, CA.

2002 Principal Investigator, NIH Res. Grant 1 R43 AI52519, $550,000 direct costs *Novel Macrolide Antibiotics*

2002 Program Chairman, Biotechnology of Microbial Products Meeting, Honolulu, HI.

2001 Principal Investigator, NIH Res. Grant 1 R43 CA93100, $100,000 direct costs *Epothilone Analogs by Genetic Engineering.*

2001 Principal Investigator, NIH Res. Grant 1 R43 AI50305, $396,232 direct costs *Anti-Infectives with Multi-domain Ribosomal Binding*

2001 Convenor, *Symposium on Genetics of Antibiotic Biosynthesis*, ISBA '01 Meeting, Vancouver, BC.

2000 Principal Investigator, NIH Res. Grant, 2 R44 CA79228 , $789,464 direct costs *Heterologous Production of Epothilone.*

2000 Charles Thom Award, Society for Industrial Microbiology

1999-2004 Lecturer, Amer. Chem. Soc. Course on *Anibiotics.*

1999-2002 Section Editor, *Current Opinion in Investigational Drugs*

1999 Convenor, *Symposium on Genetics of Antibiotic Biosynthesis*, ISBA '99 Meeting, Heraklion-Sissi, Crete.

1998 Convenor, *Seminar on Hybrid Polyketides*, GIM '98 Meeting, Jerusalem, Israel.

1998 Convenor, *Symposium on Surveillance of Antibiotic Resistance*, NMHCC Conference on Antibiotic Resistance, Atlanta, GA.

1997 Convenor, *Seminar on Polyketides*, Society for Industrial Microbiology Annual Meeting, Reno, NV.

1996 Convenor, *Seminar on Antibiotic Resistance*, Society for Industrial Microbiology Annual Meeting, Research Triangle Park, NC.

1995-2000 Editorial Board, Journal of Bacteriology

1995 Convenor, *Seminar on Antibiotic Biosynthesis and Resistance*, ASM Annual Meeting, Washington, D.C.

1994-1997 Director, Midwest Prokaryotic Molecular Genetics and Physiology Club

1994 Convenor, *Seminar on Genetics of Antibiotic Biosynthesis*, 34th Interscience Conf. on Antimicrobial Agents and Chemotherapy, Orlando FL.

1993 Ad-hoc Member, NIH IRG Study Section, Biorganic and Natural Products Chemistry

1992-1993 ASM Foundation Lecturer

1992-1995 Co-Organizer & Trainer, NIH Training Grant 5T32GM0839 in Biotechnology to University of Chicago

1992-1995 Post-doctoral Advisor to two Abbott Scientists on NIH RO1-GM46696.

1992-1995 Principal Investigator, NIH Res. Grant, RO-1 GM46696, $464,105 direct costs *Genetic Engineering of Rapamycin Analogs*

1992 Chairman's Award, Abbott Laboratories

1990 - 1998 Reviewer of Research Grants for National Science Foundation, National Science & Engineering Research Council (Canada), Medical Research Council (Canada)

1987- Reviewer for Following Journals:
Antimicrobial Agents and Chemotherapy, Applied Microbiology and Biotechnology, Biochemica and Biophysica Acta, Biochemistry, Chemistry and Biology, Gene, Journal of the American Chemical Society, Journal of Bacteriology, Journal of General Microbiology, Molecular and General Genetics, Molecular, Microbiology, Nature/Biotechnology, Peptide Research, Science, Tetrahedron

1987 Convenor, *Seminar on Antibiotic Biosynthesis*, Society for Industrial Microbiology Annual Meeting, Baltimore, MD.

1975-1977 NIH Institutional Research Grants, New York University

1970-1972 National Institutes of Health Postdoctoral Research Fellowship

1968-1970 University of California Graduate Fellowship

1966-1968 Scholar of the Province of Quebec

PUBLICATIONS

Tang, L., L. Chung, J. R. Carney, and L. Katz. 2005. *Generation of Novel Epothilones from Genetic Engineering of a Heterologously Expressed Polyketide Synthase in Myxococcus xanthus.* J. Antibiotics. (submitted).

Katz, L., and G. Ashley. 2005. Translation Inhibitors: Macrolides. Chem. Rev. (in press).

Ward, S. L., Z. Hu, A. Schirmer, R. Reid, W. P. Revill, C. D. Reeves, O. V. Petrakovsky, S. D. Dong, and L. Katz. 2004. *The chalcomycin biosynthesis gene cluster from Streptomyces bikiniensis: novel features of an unusual ketolide through expression of the chm PKS in Streptomyces fradiae.* Antimicrob. Agents Chemother. 78:4703-4712.

Rodriguez, E., S. L. Ward, H. Fu, W. P. Revill, R. McDaniel, and L. Katz. 2004. *Engineered biosynthesis of 16-membered macrolides that require methoxymalonyl-ACP precursors in Streptomyces fradiae.* Appl. Microbiol. Biotechnol. 66:85-91.

Reeves, C. D., S. L. Ward, W. P. Revill, H. Suzuki, M. Marcus, O. V. Petrakovsky, S. Marquez, H. Fu, S. D. Dong, and L. Katz. 2004. *Production of hybrid 16-membered macrolides by expressing combinations of polyketide synthase genes in engineered Streptomyces fradiae hosts.* Chem. Biol. 11:1465-1472.

Katz, L., and D. C. Myles. 2004. Polyketide biosynthesis and drug discovery. MedChem News 14; (2):7-12.

Tang, L., S. Ward, L. Chung, J. R. Carney, Y. Li, R. Reid, and L. Katz. 2004. *Elucidating the mechanism of cis double bond formation in epothilone biosynthesis.* J. Am. Chem. Soc. 126:46-47.

Katz, L. 2003. *Sir David Alan Hopwood.* J. Ind. Microbiol. Biotechnol. 30:446-447.

Tang, L., R.-G. Qui, Y. Li, and L. Katz. 2003. *Generation of novel epothilone analogs with cytotoxic activity by biotransformation.* J. Antibiotics 56:16-23.

Arslanian, R. L., L. Tang, S. Blough, W. Ma, R. –G. Qiu, L. Katz, and J. R. Carney. 2002. *A new cytotoxic epothilone from modified polyketide synthases heterologously expressed in Myxococcus xanthus.* J. Nat. Prod. 65:1061-1065.

Katz, L. 2002. *Engineering polyketide synthases.* The ScientificWorld 2:127-129.

Reeves, C. D., L. M. Chung, Y. Liu, Q. Xue, J. R. Carney, W. P. Revill, and L. Katz. 2002. *A new substrate specificity for acyl transferase domains of the ascomycin polyketide synthase in Streptomyces hygroscopicus.* J. Biol. Chem. 277:9155-9.

Katz, L. 2001. *Polyketide Diversity* in *Macrolide Antibiotics*, W. Shonfeld and H. A. Kirst, (eds.), pp. 157-175. Birkhauser Verlag, AG, Basel.

McDaniel, R. and L. Katz, 2000. *Genetic engineering of novel macrolide antibiotics.*in Development of Novel Microbial Agents: Emerging Strategies, K. Lohner, (ed.), pp. 45-60. Horizon Scientific Press.

Volchegursky, Y., Z. Hu, L. Katz and R. McDaniel. 2000. *Biosynthesis of the anti-parasitic agent megalomicin: transformation of erythromycin to megalomicin in Saccharopolyspora erythraea.* Mol. Microbiol. 37:752-762.

Wu, K., W. P. Revill, L. Chung, L. Katz, and C. D. Reeves. 2000. *The FK520 gene cluster of Streptomyces hygroscopicus var. ascomyceticus (ATCC14891) contains genes for the biosynthesis of unusual poylyketide extender units.* Gene 251:81-90.

Julien, B., S. Shah. R. Goldman, R. Ziermann. L. Katz, and C. Khosla. 2000. *Isolation and characterization of the epothilone biosynthetic gene cluster from Sorangium cellulosum.* Gene 249:153-160.

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Katz, L, and R. McDaniel. 1999. *Novel macrolides through genetic engineering.* Med. Chem. Rev. 19:543-558.

Bryskier, A., and L. Katz. 1999. *Editorial Overview: Trends in fluoroquinolone and macrolide research.* Curr. Opin. Anti-Infect. Invest. Drugs. 1:401-402.

Hadjuk, P. J., J. Dinges, J. M. Schkeryantz, D. Janowick, M. Kaminski, M. Tufano, D. J. Augeri, A. Petros, V. Nienaber, P. Zhong, R. Hammond, M. Coen, B. Beutel,, L. Katz, and S. W. Fesik. 1999. *Novel inhibitors of Erm methyltransferases from NMR and parallel synthesis.* J. Med. Chem. 42:3852-3859.

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Stassi. D. L., S. J. Kakavas, K. A. Reynolds, G. Gunawardana,, S. Swanson,, D. Zeidner, M. Jackson, H. Liu, A. Buko, and L. Katz. 1998. *Ethyl substituted erythromycin derivatives produced by directed metabolic engineering.* Proc. Nat Acad. Sci. USA 95:7305-7309.

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Pratt, S. D., X. Xuei, A. C. Mackinnon, A. M. Nilius, D. M. Hensey-Rudloff, P. Zhong, and L. Katz. 1997. *Development of a coupled VanA/VanX assay: screening for inhibitors of glycopeptide resistance.* J. Biomolecular Screening 2:241-247.

Hu, L.T., S.D. Pratt, G. Perides, L. Katz, R.A. Rogers, and M.S. Klempner. 1997. *Isolation, cloning and expression of a 70-kilodalton plasminogen binding protein of Borrelia burgdorferi.* Infect. Immun. 65:4989-4995.

Ruan, X., D. Stassi, S. Lax, and L. Katz. 1997. *A second type-I PKS gene cluster isolated from Streptomyces hygroscopicus ATCC 29253, a rapamycin-producing strain.* Gene 203:1-9.

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Summers, R. G., S. Donadio, M. J. Staver, E. Wendt-Pienkowski, C. R. Hutchinson, and L. Katz. 1997. *Characterization of ten genes from the erythromycin biosynthetic gene cluster of Saccharopolyspora erythraea that are involved in L-mycarose and D-desosamine production.* Microbiology 143:3251-3262.

Katz, L., D. T. Chu, and K. Reich. 1997. *Bacterial Genomics and the search for novel antibiotics. in* Annual Reports in Medicinal Chemistry 32, J. A. Bristol (ed.), pp. 121-130, Academic Press.

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Lomovskaya, N, L. Fonstein, X. Ruan, D. Stassi, L. Katz, and C. R. Hutchinson. 1997. *Gene disruption and replacement in the rapamycin-producing Streptomyces hygroscopicus strain ATCC 29253.* Microbiology 143:875-883.

Kao, C. M., G. Luo, L. Katz, D. E. Cane, and C. Khosla. 1996. *Engineered biosynthesis of structurally diverse tetraketides by a trimodular polyketide synthase.* J. Amer. Chem. Soc. 118:9184-9185.

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Katz, L., and S. Donadio. 1995. *Macrolides. in* Genetics and Biochemistry of Antibiotic Biosynthesis, L.C. Vining and C.E. Stuttard, (eds.), pp. 71-104, Butterworths-Heineman.

Zhong, P., S. D. Pratt, R. P. Edalji, K. A. Walter, T. F. Holzman, A. G. Shivakumar, and L. Katz. 1995. *Substrate requirements for ErmC' methyltransferase activity.* J. Bacteriol. 177:4327-4332.

Cane, D. E., G. Luo, C. Khosla, C. M. Kao, and L. Katz. 1995. *Erythromycin biosynthesis. Highly efficient incorporation of polyketide chain elongation intermediates into 6-deoxyerythronolide B in an engineered Streptomyces host.* J. Antibiotics 48:647-651.

Lambalot, R. H., D. E. Cane, J. J. Aparacio and L. Katz. 1994. *Overexpression and characterization of the erythromycin C-12 hydroxylase, EryK.* Biochemistry 34:1858-1866.

Kao, C. M., G. Luo, L. Katz, D. E. Cane and C. Khosla. 1994. *Engineered biosynthesis of a triketide lactone from an incomplete modular polyketide synthase.* J. Amer. Chem. Soc.116:11612-11613.

Metzger, R., D. P. Brown, P. Grealish, M. J. Staver, J. Versalovic, J. R. Lupski, and L. Katz. 1994. *Characterization of the macromolecular (MMS) operon from Listeria monocytogenes.* Gene 151:161-166.

McCall, J. O., S. Kadam, and L. Katz. 1994. *A high capacity microbial screen for inhibitors of human rhinovirus protease 3C* . Bio/Technology 12:1012-1016.

Kao, C. M., L. Katz, and C. Khosla. 1994. *Engineered Biosynthesis of a complete macrolactone in a heterologous host.* Science 265: 509-512.

Brown, D. P., K. B. Idler, D. M. Backer, S. Donadio, and L. Katz. 1994. *Characterization of the genes and attachment sites for site-specific integrat ion of plasmid pSE101 in Saccharopolyspora erythraea and Streptomyces lividans.* Molec. Gen. Genet. 242:185-193.

Katz, L., S. Donadio, M. J. Staver, D. L. Stassi, S. J. Swanson, P. J. Sheldon, M. Jackson, J. M. Weber, J. O. Leung, and J. B. McAlpine. 1993. *Recombinant DNA techniques in the generation of novel macrolides.* Devel. Indust. Microbiol. 33:205-218.

Donadio, S., J. B. McAlpine, P. J. Sheldon, M. Jackson, and L. Katz. 1993. *An erythromycin analog produced by reprogramming polyketide synthesis.* Proc. Natl. Acad. Sci. USA 90:7119-7123.

Donadio, S., D. L. Stassi, J. B. McAlpine, M. J. Staver, P. J. Sheldon, S. J. Swanson, M. Jackson, C. R. Hutchinson, E. Wendt-Pienkowski, Y. -G. Wang, B. Jarvis, and L. Katz. 1993. *Recent developments in the genetics of erythromycin formation. in* Industrial Microorganisms: Basic and Applied Molecular Genetics, R. H. Baltz, G. D. Hegeman, and P. L. Skatrud (eds.), pp.257-265, Amer. Soc. Microbiol., Washington, DC.

Katz, L. and S. Donadio. 1993. *Polyketide synthesis: prospects for hybrid antibiotics*. Annu. Rev. Microbiol. 47:875-912.

Katz, L., S. Donadio, J. M. Weber, J. B. McAlpine, D. L. Stassi, M. J. Staver, J. O. Leung, S. J. Swanson, D. P. Brown, and M. Jackson. 1993. *Genetics of Macrolide Biosynthesis: towards a rational approach for the design of novel structures. in* Biotechnology, Theory and Applications 1993, pp. 302-316, W. Tien, S.-F. Chen, L. Lo, Y. T. Shyu, (eds.), Developmental Center for Biotechnology, Taipei, Taiwan.

Stassi, D., S. Donadio, M. J. Staver, and L. Katz. 1993. *Identification of a Saccharopolyspora erythraea gene required for the final hydroxlation step in erythromycin biosynthesis.* J. Bacteriol. 175:182-189.

Katz, L., and C. R. Hutchinson. 1992. *Genetic Engineering of Antibiotic Organisms. in* Annual Reports in Medicinal Chemistry 27 J. A. Bristol (ed.), pp. 129-138, Academic Press.

Taylor, A., D. P. Brown, S. Kadam, M. Maus, W. E. Kohlbrenner, D. Weigl, M. C. Turon, and L. Katz. 1992. *Expression of mature HIV-1 protease in Escherichia coli under control of the araBAD promoter.* Applied Microbiol. and Biotechnol. 37:205-210.

Donadio, S., M. J. Staver, J. B. McAlpine, S. J. Swanson, and L. Katz. 1992. *Biosynthesis of the erythromycin macrolactone and a rational approach for producing hybrid macrolides*. Gene 115:97-103.

Donadio, S., and L. Katz. 1992. *Organization of the enzymatic domains in the multifunctional polyketide synthase involved in erythromycin biosynthesis in Saccharopolyspora erythraea.* Gene 111:51-60.

Katz, L., D. P. Brown, and S. Donadio. 1991. *Site-specific recombination in Escherichia coli between the att sites of plasmid pSE211 from Saccharopolyspora erythraea.* Molec. Gen. Genet. 227:155-159.

Donadio, S., M. J. Staver, J. B. McAlpine, S. J. Swanson, and L. Katz. 1991. *Modular organization of genes required for complex polyketide synthesis.* Science 252: 675-679.

Tuan, J. S., J. M. Weber, M. J. Staver, J. O. Leung, and L. Katz. 1990. *Cloning of genes involved in erythromycin biosynthesis from Saccharopolyspora erythraea using a novel actinomycete-Escherichia coli cosmid.* Gene 90:21-29.

Paulus, T. J., J. S. Tuan, V. E. Luebke, G. T. Maine, J. P. Dewitt, and L. Katz. 1990. *Mutation and cloning of eryG, the structural gene for erythromycin O-methyltransferase from Saccharopolyspora erythraea and expression in Escherichia coli.* J. Bacteriol. 172:2541-2546.

Brown, D. P., K. B. Idler, and L. Katz. 1990. *Characterization of the genetic elements required for site-specific integration of plasmid pSE211 in Saccharopolyspora erythraea.* J. Bacteriol. 172:1877-1888.

Donadio, S., J. S. Tuan, M. J. Staver, J. M. Weber, T. J. Paulus, G. T. Maine, J. O. Leung, J. P. Dewitt, J. A. Vara, Y. -G., Wang, C. R. Hutchinson, and L. Katz. 1989. *Genetic studies on erythromycin biosynthesis in Saccharopolyspora erythraea. in* Genetics and molecular biology of industrial microbiology. C. L. Hershberger, (ed.), pp. 53-59. Amer. Soc. Microbiol., Washington, DC.

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INVITED LECTURES

GIMBM/BMP Meeting, san Diego, CA, November 2004, Invited by Kevin Reynolds

Children's Oakland Research Institute, Oakland, CA, September 2004, Invited by Stuart Smith
SIM Annual Meeting, Minneapolis, MN, August 2003, Invited by Ben Shen
Canadian Bacterial Diseases Network, Calgary, AB, April 2003, Invited by Julien Davies
Miami Winter Symposium, Miami, FL, February 2002, Invited by Sandy Black
SBS Annual Meeting, Baltimore, MD, September 2001, Invited by Jim McAlpine
SIM Annual Meeting, San Diego, CA, July 2000, Charles Thom Award Lecture
SIM Annual Meeting, San Diego, CA, July 2000, Invited by David Sherman
University of South Carolina Medical School, March 2000, Invited by Jurgen Rohr
University of California (Davis), November 1999, Invited by Ben Shen
SIM Annual Meeting, Washington, DC, August 1999, Invited by Ray Lam
University of Leicester, Leicester, UK, July 1999, Invited by Peter Williams
John Innes Centre, Norwich, UK, July 1999, Invited by Sir David Hopwood
IIR Conference on Natural Products, London, UK, June 1999, Invited by Tom Simpson
ASM Annual Meeting, Chicago, IL, May 1999, Invited by Arnold Demain
Suddath Memorial Symposium, Georgia Institute of Technology, Atlanta, GA, April 1999, Invited by Leon Zalkow
IBC Conference on Enzyme Technologies, San Francisco, CA, March 1999, Invited by David Demergian
3rd Winter Conference on Medicinal and Bioorganic Chemistry, Steamboat Springs, CO, January 1999, Invited by Les Mitscher
SIM Annual Meeting, Denver, CO, August 1998, Invited by Sir David Hopwood
NIH Rocky Mountain Laboratories, July 1998, Invited by Pamela Small
Polyketides II, sponsored by the Royal Society of Chemistry, University of Bristol, July 1998, Invited by Sir David Hopwood
GIM '98, Jersusalem, Israel, June 1998, Invited by Yair Aharonowitz
ASM Annual Meeting, Atlanta, GA, May 1998, Invited by David Sherman
Virginia Commonwealth University, Richmond, VA, January 1998, Invited by Kevin Reynolds
Chicago Medical School, North Chicago, IL, October 1997, Invited by Michael Fennewald
SIM Annual Meeting, Reno, NV, August 1997, Invited by Joan Bennett
Gordon Conference of Natural Products, Henniker, NH, July 1997, Invited by Jon Clardy
ISBA '97, Beijing, China, May 1997, Invited by J. S. Chiao
Biotechnology of Microbial Products, Williamsburg, VA, April 1997, Invited by Dick Hutchinson
University of Minnesota, Minneapolis, MN, February 1997, Invited by Gary Dunny
BioEast '97, Washington, DC, January 1997, Invited by William Timberlake
Southern Great Lakes Region of SIM Annual Meeting, Chicago Heights, IL, October 1996, Invited by Charles Hershberger
SIM Annual Meeting, Research Triangle Park, NC, August 1996, Invited by Mark Finkelstein
Workshop of Biosynthetic Approaches to Natural Product Production, Bethesda, MD, June 1996, Invited by Gordon Cragg
Polyketides Meeting, sponsored by the Royal Society of Chemistry, University of Bristol, April 1996, Invited by Sir David Hopwood
University of Leicester, Leicester, UK, April 1996, Invited by Peter Williams
Technion, Haifa, Israel, December 1995, Invited by Yuval Shoham
Pacific Rim Chemical Society, Honolulu, Hawaii, December 1995, Invited by David Sherman
Tel Aviv University, December 1995, Invited by Gerald Cohen
University of Groningen, The Netherlands, December 1995, Invited by Lubbert Dijkhuizen
Beijerinck Centennial Symposium, The Hague, Netherlands, December 1995, Invited by W.A. Scheffers
Canadian Society for Microbiologists Annual Meeting, June 1995, Invited by Leo Vining
ASM Annual Meeting, Washington, DC, May 1995, Invited by Douglas Eveleigh
34th ICAAC Meeting, Orlando, FL, October 1994, Invited by Jim McAlpine
ISBA'94, Moscow, Russia, July 1994, Invited by Dick Hutchinson
U.S.-Japan Seminar on Biosynthesis of Natural Products, Tokyo, Japan, June 1994, Invited by David Cane

Louisiana State University Medical Center, Shreveport, LA, February 1994, Invited by Peter Zuber
University of Minnesota, Minneapolis, MN, November 1993, Invited by David Sherman
Gordon Conference on Enzymes, Coenzymes and Metabolic Pathways, Meriden, NH, July 1993, Invited by Ming-Daw Tsai
5th Society of Chinese Bioscientists in America Intl Symposium, Baltimore, MD. June 1993, Invited by Shing Chang
Ohio State University, Columbus, OH, April 1993, Invited by Anthony Young
3rd SIM Intl Conference on the Biotechnology of Microbial Products, Rohnert Park, CA, April 1993, Invited by Arnold Demain
Stanford University, Stanford, CA, March 1993, Invited by Chaitan Khosla
17th Fungal Genetics Conference, Asilomar, CA, March 1993, Invited by Deepak Bhatnagar
Chicago Medical School, North Chicago, IL, January 1993, Invited by Michael Fennewald
ASM Conference on Genetics of Industrial Microorganisms, Bloomington, IN, October 1992, Invited by Richard Baltz
University of Wisconsin, Madison, WI, September 1992, Invited by Glenn Chambliss
3rd Pacific Rim Conference on Biotechnology, Taipei, Taiwan, August 1992, Invited by Shing Chang
ASM Annual Meeting, New Orleans, LA, May 1992, Invited by Deepak Bhatnagar
University of Georgia, Athens, GA, May 1992, Invited by Philip Youngman
ASM Foundation Lecture, ASM Eastern NY Branch, Albany, NY, April 1992, Invited by Karim Hechemy
Stanford University, Stanford, CA, April 1992, Invited by Stanley Cohen
Children's Oakland Research Institute, Oakland, CA, April 1992, Invited by Stuart Smith
203rd National ACS Meeting, San Francisco, CA, April 1992, Invited by David Hopwood
Midwest Prokaryotic Genetics and Microbial Physiology Club, Chicago, IL, January 1992, Invited by Philip Matsumura
University of Illinois at Chicago, Chicago, IL, October 1991, Invited by A.M. Chakrabarty
202nd Annual ACS Meeting, New York, N.Y., August 1991, Invited by Leo Vining
ISBA'91, Madison, WI, August 1991, Invited by Gene Seno
ASM Conference on Biotechnology, New York, NY, June 1991, Invited by Dick Hutchinson
Harvard University, Cambridge, MA, May 1991, Invited by Richard Losick
Brown University, Providence, RI, February 1991, Invited by David Cane
Chicago Medical School, North Chicago, IL, January 1991, Invited by Michael Fennewald
Engineering Foundation Conferences on Progress in Recombinant DNA Technology and Applications, Potosi, MO, June 1990, Invited by Janet Westpheling
ASM Annual Meeting, Anaheim, CA, May 1990, Invited by Arnold Demain
UCLA Symposium of Molecular Biology of Streptomyces, Frisco, Colorado, January 1990, Invited by Mervyn Bibb
Northern Illinois University, May 1989, Invited by Patricia Vary
ASM Conference on Genetics of Industrial Microorganisms, Bloomington, IN, October 1988, Invited by Richard Losick
SIM Annual Meeting, Chicago, IL, August 1988, Invited by Arnold Demain
ACS Annual Meeting, New Orleans, LA, August 1987, Invited by Dick Hutchinson
UCLA-SIM Colloqium on Streptomycetes, Lake Tahoe, CA, March 1987, Invited by Mervyn Bibb
New York University, New York, NY, January 1987, Invited by Guenther Stotzky
University of Leicester, Leicester, UK, September 1986, Invited by Peter Williams
Harvard University, Cambridge, MA, June 1986, Invited by Richard Losick
New York University, New York, NY, December 1974, Invited by Guenther Stotzky
University of California, La Jolla, CA, September 1970, Invited by Don Helinski
Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, July 1970, Invited by Jeffrey Miller
Gordon Conference on Biological Regulatory Mechanisms, Tilton, NH, July 1970, Invited by Ellis Englesberg

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Biosynthesis of Polyketides in Heterologous Hosts

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INTRODUCTION

The explosive growth in the number of cloned and sequenced genes has led to an enhanced need for robust heterologous gene expression methods. This need exists, in part, because the proteins encoded by these numerous genes of interest promise to have an important impact in areas ranging from basic biochemical and biophysical research to the practical use of proteins as pharmaceuticals, animal health products, and industrial enzymes. Notwithstanding the enormous efforts that have gone into the development of the heterologous-expression toolbox, the production of a desired protein in a heterologous host remains an empirical and often unpredictable process.

Although there is no universal solution for heterologous protein production, certain generalizations can be made. A few prokaryotic and eukaryotic systems such as *Escherichia coli*, *Saccharomyces cerevisiae*, Sf9 insect cells, and Chinese hamster ovary (CHO) cells have emerged as choice hosts due to their simplicity of use, excellent growth characteristics, and a plethora of readily accessible genetic tools. In turn, this has fueled studies aimed at understanding the cellular machinery responsible for protein synthesis, posttranslational modification, protein folding, trafficking, and degradation in these model host cells. Another feature common to good heterologous hosts is the availability of good fermentation protocols that maximize protein productivity, especially those in which cell growth can be decoupled from recombinant gene expression. Finally, it is generally understood that a balance exists between product quality and quantity, regardless of the host used. High levels of gene expression are often accompanied by phenomena such as inclusion body formation, increased amino acid misincorporation, incomplete or inaccurate posttranslational modification, reduced recombinant cell line stability, and a range of other features associated with a greater metabolic burden as a result of heterologous protein production.

In most cases, the ultimate goal of heterologous gene expression is to produce the desired protein in reagent quantities. Over the past 20 years, however, there has been a growing interest in the potential for harnessing the intrinsic metabolic activity of proteins in heterologous hosts. Multiple genes are often coexpressed in metabolic engineering, where the goal is not simply to synthesize large quantities of the target proteins themselves but to optimally interweave their activities among each other as well as among functional protein networks in the host. Examples of metabolic engineering include the bacterial biosynthesis of indigo (19) and the conversion of 3-dehydroshikimic acid, a key intermediate in aromatic amino acid metabolism, into a variety of value-added products such as vanillin (47).

Over the past decade, the study of bioactive natural-product biosynthesis has benefited significantly from the use of heterologous hosts. Notably, the rapid growth in understanding and manipulating polyketide biosynthesis closely parallels developments in the ability to reconstitute these multistep catalytic processes in genetically (and now genomically) friendly heterologous hosts. This review focuses on the development of heterologous expression systems for polyketide synthases (PKSs), and discusses their impact on the field of natural-product biosynthesis and drug development. Reconstitution of polyketide biosynthesis in heterologous hosts demands that large multien-

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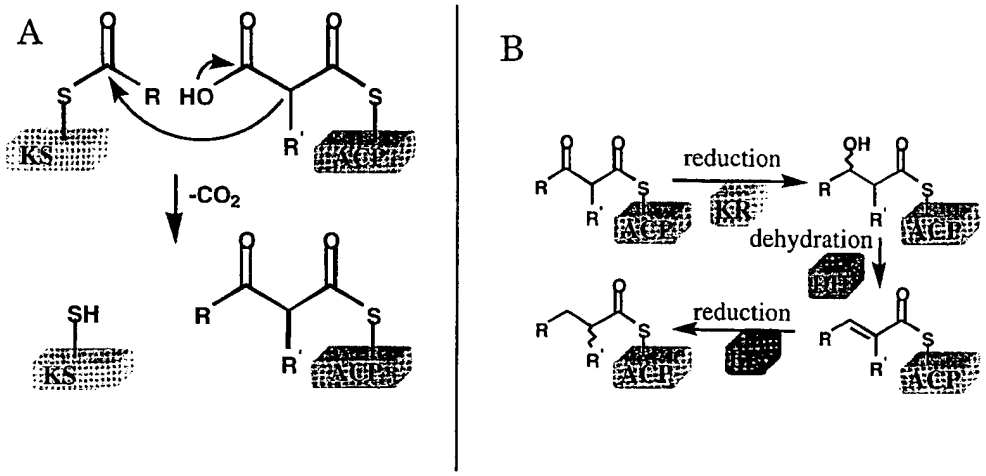

FIG. 1. Key reactions and catalysts in polyketide biosynthesis. (A) The decarboxylative condensation reaction that defines a polyketide. The electrophile is attached to the ketosynthase (KS), whereas the nucleophile is attached to an ACP. (The only known exceptions to this rule are the chalcone synthase-like PKSs [see Fig. 3], where the nucleophile remains attached to CoA) (B) In addition to the above-mentioned C—C bond-forming reaction, PKSs catalyze all, some, or none of the following reactions. The β-carbonyl generated on C—C bond formation can be reduced by an NADPH-dependent enzyme called a ketoreductase (KR). The resulting alcohol can be dehydrated by a dehydratase (DH). The resulting olefin can be hydrogenated by another NADPH-dependent enzyme called an enoylreductase (ER). Other, less commonly occurring reactions such as C-methyl transfers (28) (see also Fig. 4) are not shown.

zyme assemblies be functionally expressed, their posttranslational modification needs be adequately met, their substrates be available in vivo in reasonable quantities, and the producer cell be protected against the toxicity of the biosynthetic products. Two particular heterologous hosts will be the focus of most of our discussion—*Streptomyces coelicolor* and *Escherichia coli*. However, the relative merits of a variety of other heterologous hosts will also be discussed.

WHY POLYKETIDES?

Polyketides, as the name implies, are synthesized from repetitive condensation reactions that link small carbon precursors (typically, 2- and 3-carbon acyl groups derived from coenzyme A (CoA) thioesters) (Fig. 1A) (63). The process is similar in many respects to bacterial and mammalian fatty acid synthesis. In fact, PKSs are classified into type I or II categories based on how closely they mimic the architecture of type I (vertebrate) or type II (bacterial and plant) fatty acid synthases (35). However, unlike fatty acids, the structures of polyketides are far more diverse due to variations in the fatty acid synthesis theme (Fig. 1B) and post-PKS modifications. This structural diversity is also reflected in diversity in their biological modes of action. A number of polyketides have been clinically approved as drugs for treating disorders such as infections, cancer, cardiovascular diseases, and inflammation. Approximately two-thirds of the known bioactive polyketide natural products originate from the actinomycetes. Other major microbial sources of polyketides include the myxobacteria and filamentous fungi. The exact role of polyketides in the life cycles of producing organisms remains unknown, but these secondary metabolites are presumably synthesized to ward off competing microbes during periods of nutrient limitation. Some polyketides are also synthesized as spore pigments (14, 55).

Figure 2 shows examples of familiar polyketides and their representative biological sources. It also highlights differences in the architectures of PKSs that synthesize the carbon skeletons of these natural products. Four broad architectural varieties of PKSs have been isolated thus far from microbes. The first category of PKSs is perhaps the simplest and actually resembles plant PKSs such as the chalcone synthase. An example is shown in Fig. 3 (24). This is the only variety of PKS in which the nucleophilic group involved in each C—C bond-forming reaction is attached to a CoA instead of an acyl carrier protein (ACP). Although the primary amino acid sequences of these PKSs are only weakly related to those of other PKSs, X-ray crystallographic analysis (22) and site-directed mutagenesis (38) of a prototype of this family, a plant chalcone synthase, have clearly established the close evolutionary connection between these PKSs and others. Fungal PKSs represent a second subclass of PKSs that closely resemble vertebrate (type I) fatty acid synthases. The proteins are multidomain and act in an iterative fashion. An example is the lovastatin LNKS, which catalyzes the formation of dihydromonacolin, the polyketide precursor for lovastatin biosynthesis (Fig. 4) (41). Bacterial PKS systems with an architectural relationship to type II fatty acid synthases represent a third PKS category. Also acting in an iterative fashion, these multienzyme systems produce aromatic polyketide products. However, individual active sites occur as distinct polypeptides rather than as domains within a single multifunctional polypeptide. An example is the doxorubicin PKS, which synthesizes the tetracyclic skeleton of this anthracycline antibiotic (Fig. 5) (2). Finally, a fourth category of PKSs, known as modular PKSs, is exemplified by the deoxyerythronolide B synthase (Fig. 6) (13, 17). Here, polyketide biosynthesis proceeds in a processive fashion on a very large megasynthase in which each active site is used once during the overall catalytic cycle.

There are several features associated with microbial polyketide biosynthesis that make these pathways particularly well

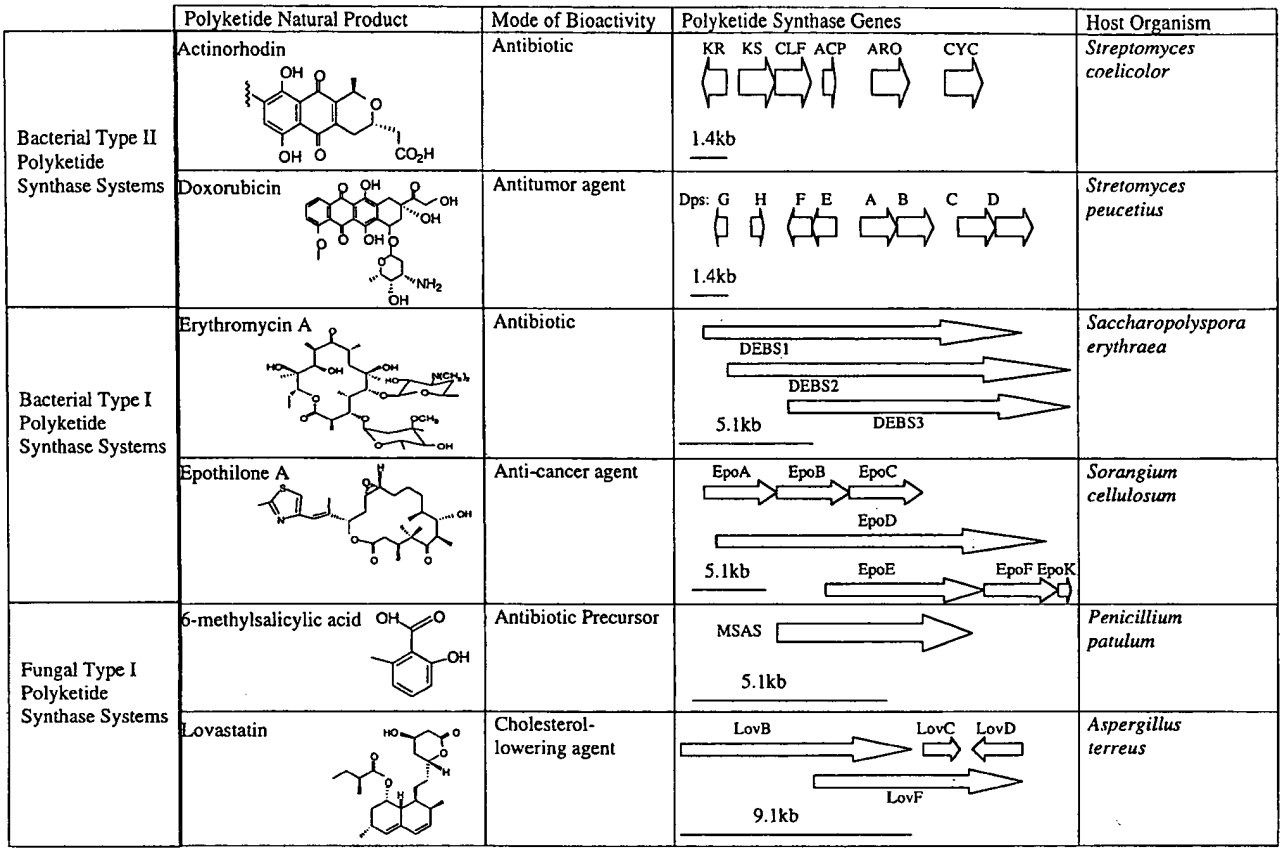

FIG. 2. Representative polyketide natural products and PKS systems. This figure illustrates the diversity in polyketide natural products and the PKSs that catalyze their formation. The block arrows represent ORFs encoding different PKS components. The given scales indicate approximate ORF sizes, although individual catalytic domains are not shown. In general, the larger proteins encode several catalytic domains whereas the smaller polypeptides possess a single enzymatic activity. Protein abbreviations: KS, ketosynthase; CLF, chain length factor; KR, ketoreductase; ARO, aromatase; CYC, cyclase; Dps, doxorubicin polyketide synthase; DEBS, deoxyerythronolide B synthase; MSAS, methyl salicylic acid synthase.

suited for heterologous expression. First, PKSs are remarkably similar in primary sequences and (by inference) tertiary structures, giving hope that, as improved heuristics for heterologous expression in a given host emerge, they can be applied with greater confidence to newer systems. Second, in all known bacterial and fungal examples, PKS genes have been found to exist as gene clusters (together with transcriptional regulators and self-resistance genes), a feature that greatly simplifies their genetic isolation as well as heterologous expression (at least in bacteria, where multigene operons can be constructed). Third, polyketides are naturally produced as secondary metabolites, suggesting that they can be readily produced in two-stage fermentations in which cell growth can be decoupled from product formation. Fourth, an extraordinary diversity of chemotypes can be synthesized by the PKS paradigm from a relatively small subset of intracellular precursors such as acetyl-CoA, propionyl-CoA, malonyl-CoA, and methylmalonyl-CoA. Moreover, each of these precursors can be derived via multiple metabolic routes from exogenously available carbon sources, thereby presenting a range of options for any potential heterologous host. Finally, as the time lines for converting pharmacologically interesting leads into clinically useful molecules continue to shrink, the earlier paradigm of developing the biology of new microbes for every new polyketide natural product of interest is no longer practical. Lateral transfer of a pathway of interest into a well-developed surrogate host therefore becomes an attractive alternative, both with respect to overproducing the parent natural product itself and for generating novel analogs via biosynthetic engineering. Notwithstanding these advantages, there are several unique characteristics of PKSs that make heterologous expression a definite challenge. Notable PKS characteristics include their relatively large size (100 to 10,000 kDa), the high G+C content of many PKS genes (especially those coming from the actinomycetes, where the G+C content typically exceeds 70%), their requirement for posttranslational modifications, and the relatively rudimentary understanding of the special regulatory and metabolic features associated with the transition from primary to secondary metabolism in any organism.

FACTORS THAT INFLUENCE HETEROLOGOUS PRODUCTION OF POLYKETIDES

As is now clear from several studies involving reconstituted PKS systems (reviewed in reference 42), PKSs are soluble,

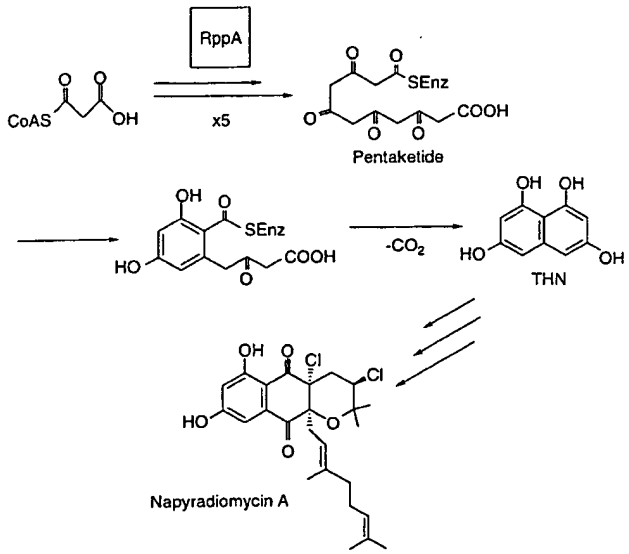

FIG. 3. Chalcone synthase-like PKS. The RppA PKS iteratively condenses five malonyl-CoA units, which then cyclize to form 1,3,6,8-tetrahydroxynaphthalene (THN). THN most probably represents an intermediate in the production of antibiotics such as napyradiomycin A. RppA resembles a ketosynthase (KS) domain in other PKS paradigms. However, in this case, RppA condenses malonyl-CoA units directly as opposed to accepting a malonyl unit from an ACP.

cytosolic multienzyme systems that do not require any intracellular substructure or organelle to maintain activity. Individually purified protein components (or in some instances proteins purified as heterodimers) can be mixed in vitro to yield PKS activity. While not completely ruling out the need for additional cellular machinery to enhance PKS activity, these experiments indicate that, if introduced into a foreign cellular host, the DNA encoding PKS genes, when actively expressed, should support polyketide production provided that the required substrates are available. Below we review the important considerations for reconstituting polyketide pathways in a heterologous host.

Posttranslational Modification

It has long been known that the active site of an ACP involves the thiol moiety of a 4′-phosphopantetheine group that is covalently attached to a conserved serine residue in the polypeptide (79, 83). This pantetheinyl group is posttranslationally derived from intracellular CoASH (Fig. 7) (18). Until recently, very little was known about the enzymatic basis for this modification. Over the past few years, the work of Walsh and coworkers has been instrumental in identifying an evolutionarily related superfamily of enzymes, the phosphopantetheinyl transferases (PPTases), that catalyze this reaction (44, 45). Although these enzymes are known to exist in all organisms except perhaps for the archaea (which do not make fatty acids or polyketides), individual members of this superfamily have significantly distinct configurations and substrate preferences. For example, the *E. coli* genome contains at least three different PPTase genes (44), each existing as an individual open reading frame (ORF), whereas in *Saccharomyces cerevisiae* the PPTase that recognizes the cognate fatty acid ACP is a distinct domain within one of the fatty acid synthase subunits (44). Likewise, the *E. coli* PPTase, which ordinarily pantetheinylates its fatty acid ACP, has relatively tight selectivity for its cognate substrate (44). In contrast, the *sfp* gene product, which is part of the surfactin biosynthetic gene cluster in *Bacillus subtilis*, is among the most tolerant PPTase discovered to date and can effectively modify ACPs from all PKS subclasses as well as related peptidyl carrier protein and aryl carrier protein domains from nonribosomal peptide synthetases (NRPSs) (11, 32, 40, 44, 69). (Although a thorough discussion of NRPSs is outside the scope of this review, NRPSs will be occasionally referred to below, given the close relationships between these systems. For further details, the reader is directed to recent reviews on the subject [10, 61].) Therefore, in deciding on a strategy for heterologous PKS gene expression, the choice of a partner PPTase is an important consideration, from both the substrate specificity and the gene regulation perspectives. Depending on the host and expression system chosen, this posttranslational modification may or may not happen.

Substrate Availability

Once functionally expressed and posttranslationally modified within a cellular host, the PKS will require a substrate pool to draw upon for polyketide production. PKSs are known to utilize a broad range of substrates including acetyl-CoA, propionyl-CoA, isobutyryl-CoA, isovaleryl-CoA, malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, propylmalonyl-CoA, and hydroxymalonyl-CoA (or its methylated counterpart, methoxymalonyl-CoA) (37). When the substrates are chiral, such as methylmalonyl-CoA, the corresponding acyltransferases exhibit strict stereospecificity (52). The α-carboxylated substrates (e.g., malonyl-CoA and methylmalonyl-CoA) are sources of extender units, whereas neutral substrates such as acetyl-CoA are sources of primer units for polyketide chain synthesis. Finally, many "hybrid" natural products are derived from the tandem action of PKSs and NRPSs (9). These multifunctional enzymes utilize an even broader range of substrates, including carboxylic acids such as *p*-aminobenzoic acid, 3-amino-5-hydroxybenzoic acid, cyclohexenoyl carboxylic acid, and dozens of α- and β-amino acids. Preactivated forms (e.g., CoA thioesters) of these free acids are not required; rather, the acids are activated in situ by ATP-dependent adenylation domains that are intrinsic components of NRPS modules (61).

From the above shortlist, it can be appreciated that a good heterologous host must be endowed with the ability to synthesize an impressive range of substrates (in addition, of course, to NADPH and ATP, which are routinely available). Moreover, the supply of these precursors must be coordinately regulated with polyketide biosynthesis so as to ensure availability when required while at the same time avoiding imbalances in tightly controlled metabolite pools (such as those corresponding to various CoA derivatives). Although this might appear to be a formidable problem for the metabolic engineer, several simplifying factors deserve to be noted. (Some of these points are also elaborated below.) First, many polyketide natural products are derived from a subset of acetyl-CoA, propionyl-CoA, malonyl-CoA, and (2*S*)-methylmalonyl-CoA alone. Therefore, the ability of a candidate host to supply just these four metabolites can make it an attractive environment for heterologous

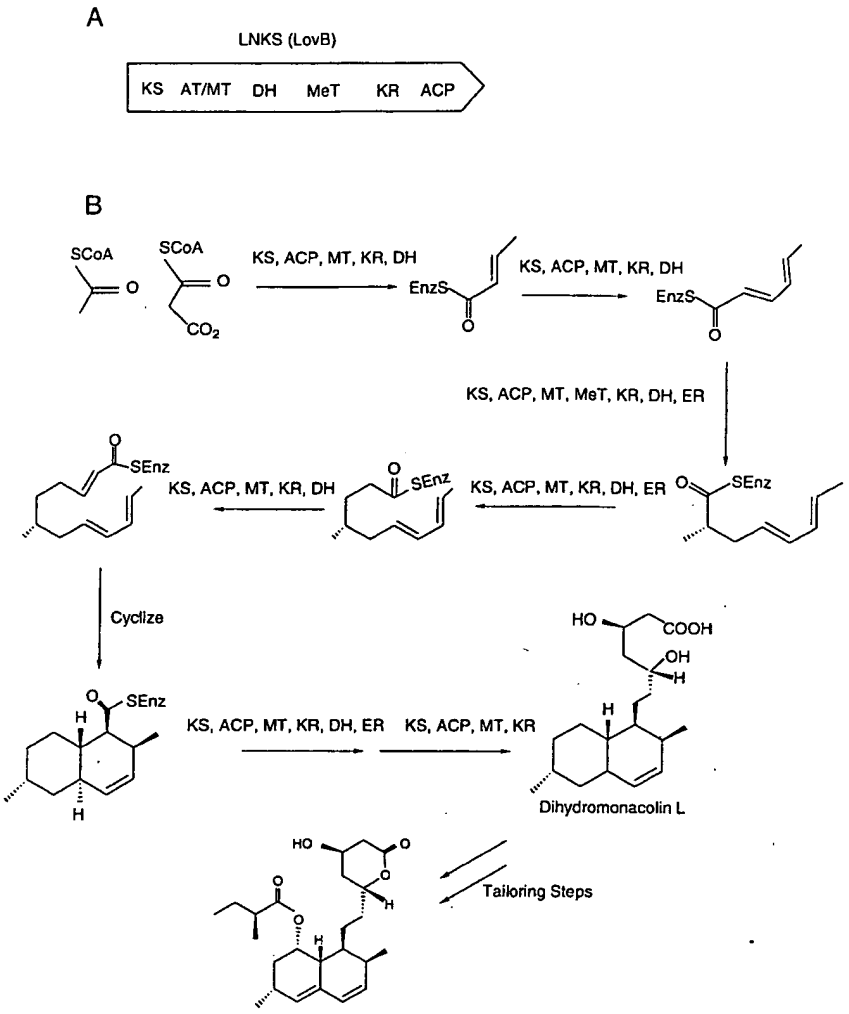

FIG. 4. Fungal type I PKS. (A) The lovastatin nonaketide synthase (LNKS; LovB) protein, with its individual enzymatic domains denoted within the PKS. KS, ketosynthase; AT/MT, acetyl/malonyl transferase; DH, dehydratase; MeT, methyltransferase; KR, ketoreductase; ACP, acyl carrier protein. (B) LNKS works in an iterative fashion to produce dihydromonacolin L. The LNKS catalyzes a decarboxylative condensation, and the reaction product (the polyketide chain) then transfers to a new LNKS unit. The reaction arrows indicate condensation reactions between an extender malonyl unit and either a priming acetate unit or the growing polyketide chain (eight condensations in total). The KS domain accepts the starter acetate unit or a growing polyketide chain and catalyzes the condensation with a malonyl unit loaded onto the ACP domain by the AT/MT. The level of reduction applied for a particular iteration is denoted near the arrows. In this case, a separate lovastatin PKS protein (not shown) provides the enoylreductase (ER) activity.

PKS expression. Second, since multiple metabolic routes are known to exist for many of these precursors (Fig. 8), a given host needs to be endowed with only one such pathway in order to facilitate polyketide synthesis. For example, at least four pathways to (2*S*)-methylmalonyl-CoA are known to exist in bacteria (7, 8, 16, 33, 36, 71, 87). (It should be noted that, from a quantitative viewpoint, not all biosynthetic pathways are energetically or kinetically equivalent.) Third, the broad specificity of certain enzymes that synthesize some of the above metabolites facilitates intracellular production of more than one PKS substrate from a given pathway, depending on the exogenous supply of carboxylic acids. For example, a single enzyme, malonyl-CoA synthetase, can be used to synthesize numerous α-carboxylated CoA thioesters from their corresponding exogenously supplied 1,3-dicarboxylic acids (N. Pohl and C. Khosla, unpublished data). Fourth, the modularity of many PKSs makes it feasible to tailor their substrate preferences to the available intracellular pool of CoA thioesters in a given host (48, 65, 73). A similar approach can also be used to constrain the substrate range of NRPSs, if desired. Fifth, the biosynthetic pathways for relatively uncommon substrates, such as hydroxymalonyl-CoA or 3-amino-5-hydroxybenzoic acid, are often encoded as part of PKS gene clusters that utilize these substrates (1, 75). Therefore, these auxiliary genes can also be harnessed for heterologous expression in very much the same way as the target PKS genes. Finally, since the actinomycetes are the most prolific producers of polyketides, the complete genome sequence of *Streptomyces coelicolor* (http://www.sanger.ac.uk

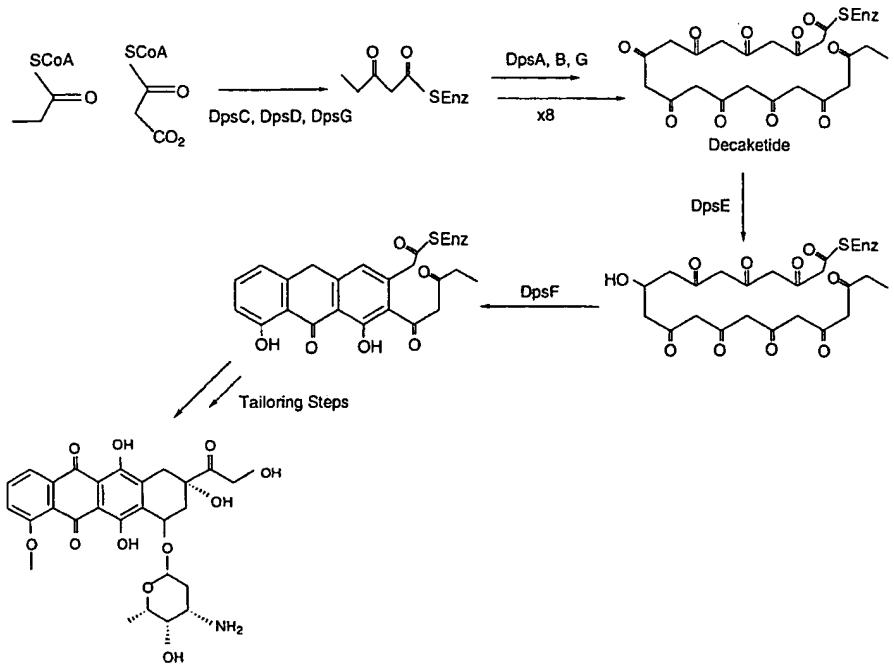

FIG. 5. Bacterial type II PKS. The doxorubicin polyketide synthase (Dps) proteins iteratively condense one propionyl-CoA unit and nine malonyl-CoA units. Initially, DpsC, DpsD, and DpsG (analogous to the KS, CLF, and ACP) catalyze a diketide formation between propionyl-CoA and malonyl-CoA. The DpsA, DpsB, and DpsG (again, analogous to the KS, CLF, and ACP) domains catalyze eight more condensation reactions, starting with the previous diketide and adding successive malonyl units to eventually produce a decaketide. The DpsE domain (encoding a KR activity) then reduces the polyketide chain before DpsF cyclizes the chain. DpsH appears to provide another cyclase activity.

/Projects/S_coelicolor/), together with its concomitant development as a heterologous host of choice for PKS gene expression, makes the task of the metabolic engineer considerably easier. Orthologs of many known precursor biosynthetic enzymes have already been identified in the genome (unpublished results), and many more can be expected to emerge as functional genomic approaches are applied to investigate the metabolome of this genetically friendly bacterium.

Other Intracellular Factors

As mentioned above, coincubation of active PKSs and their substrates is adequate for polyketide formation. However, the surrounding cellular environment may contribute to the optimization of this process in many ways. For example, folding and subsequent quaternary assembly of PKSs may benefit from the activity of many known and as yet uncharacterized chaperones. It is unlikely that PKS folding or assembly is absolutely dependent on the presence of dedicated chaperones, since thus far (i) no mutants defective in polyketide biosynthesis have been shown to have mutations that map onto putative chaperone genes, (ii) no chaperone-like genes have been identified within PKS gene clusters, and (c) PKSs have been heterologously expressed in a diverse range of microbial hosts, including *E. coli, Streptomyces, Aspergillus*, and yeast. However, the search for auxiliary factors that enhance PKS gene expression in vivo has only just begun. It is likely that quantitative aspects of intracellular PKS activity are influenced by factors that affect the stability of >50-kb transcripts, translational processivity of >30-kb ORFs, translational coupling of ORFs encoding subunits that must assemble in 1:1 stoichiometric ratios, cotranslational and posttranslational protein folding, and proteolytic degradation. An understanding of the mechanistic basis for these activities could have important implications for heterologous PKS expression.

Transmembrane Transporters

Given the potential cytotoxicity of most bioactive polyketides, transmembrane proteins are required for their export. Although putative export proteins (often ATP binding cassette transporter homologs) are often found associated with PKS gene clusters, very little is known about their mechanism or selectivity. For example, inactivation of the gene encoding the actinorhodin exporter in *S. coelicolor* leads to intracellular accumulation of this isochromanequinone antibiotic (20). However, even in a mutant lacking the entire actinorhodin gene cluster, a number of novel polyfunctional aromatic compounds have been produced and are efficiently secreted into the extracellular medium (56). While the possibility of passive diffusion of these compounds cannot be excluded, the lack of observed toxicity due to polyketide production (even in cases where antibacterial activity of the products has been demonstrated) suggests that other relatively tolerant transporter proteins might be encoded by the *S. coelicolor* genome. Here, too, functional genomic approaches may be useful in providing clues that will lead to the identification of these transporters.

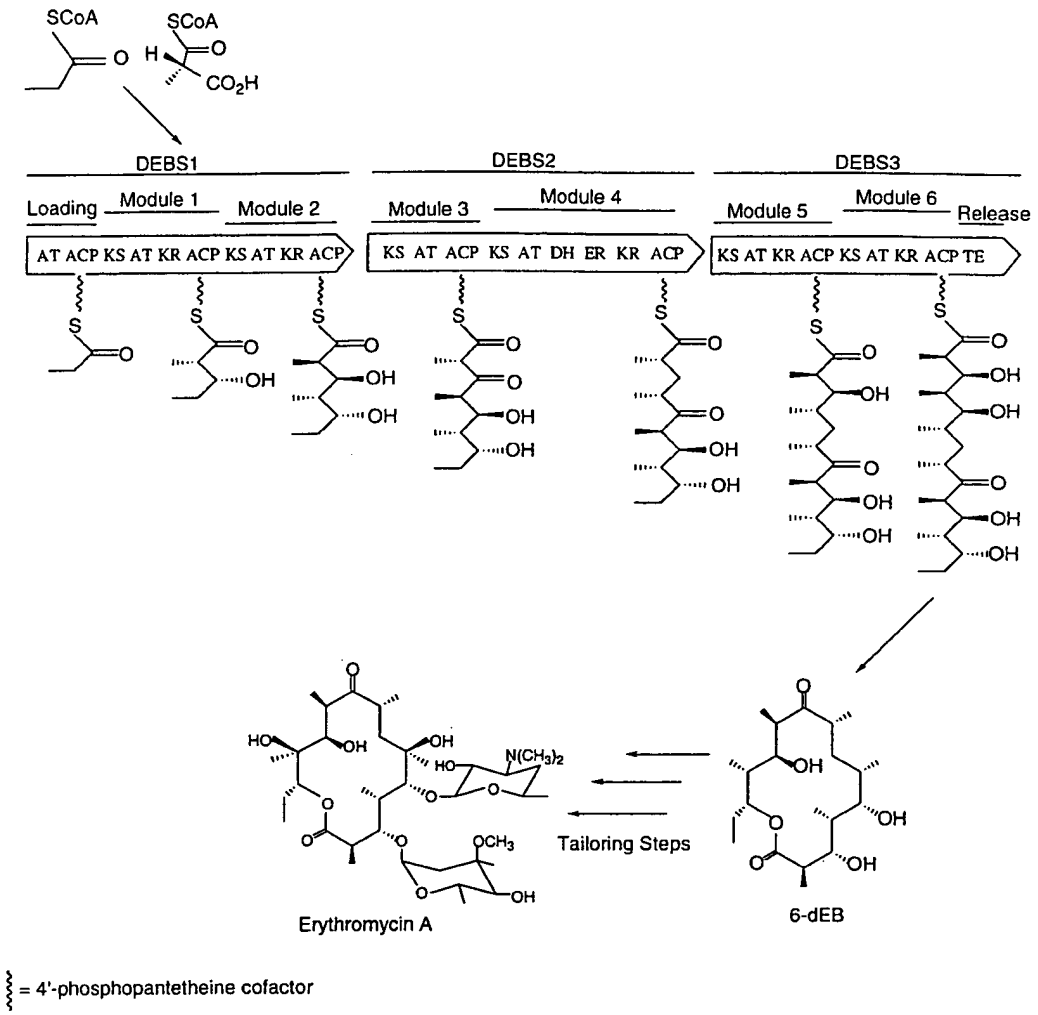

FIG. 6. Bacterial type I PKS. The deoxyerythronolide B synthase (DEBS) system catalyzes the formation of the erythromycin derivative 6-deoxyerythronolide B (6-dEB) by processively combining one propionyl-CoA primer unit and six methylmalonyl-CoA extender units. Each DEBS protein contains two modules; modules represent a physical location for a Claisen-like condensation reaction. For example, once loaded onto the KS of module 1 by the loading acyl transferase (AT) and ACP domains, propionyl-CoA condenses with a methylmalonyl unit loaded onto module one ACP by the module 1 AT domain. After the reaction takes place, reductive domains, specific for each module, reduce the resulting ketone group. The chain then passes in a processive manner from module 1 ACP (via the phosphopantetheine arm) to the KS domain of the next module. In this fashion, the polyketide chain grows and diversifies before being released and cyclized by the C-terminal thioesterase (TE) domain.

Post-PKS Polyketide Modification

The formation of biologically active polyketides sometimes requires the activity of various "tailoring" enzymes that act on the PKS-derived intermediate to yield the final natural product. Tailoring enzymes are evolutionarily diverse entities that commonly include cyclases, group transferases (e.g., *C*-, *O*-, and *N*-methyltransferases, glycosyltransferases, and acyltransferases), NADP(H)- or FAD(H)-dependent oxidoreductases, and cytochrome P450-type oxygenases. These enzymes are invariably encoded by genes adjacent to PKS genes and can therefore be readily cloned. In most cases, heterologous expression of these monofunctional enzymes is relatively straightforward. Sometimes, however, cosubstrate availability can be an issue. For example, glycosyltransferases associated with polyketide pathways often utilize specialized TDP-deoxysugars, themselves the products of multistep biosynthetic pathways (26, 66, 80). As with PKS genes, the clustering of the genes responsible for TDP-sugar biosynthesis facilitates their expression in heterologous hosts (64).

Self-Resistance

If biologically active polyketides are to be heterologously produced, a final consideration would be the need for a resistance mechanism to inhibit the effect of the natural product on the heterologous host. Fortunately again, self-resistance genes invariably exist within the natural PKS gene cluster (15, 20, 25). However, coexpression of these genes adds another level of complexity to the heterologous production of polyketides and can be particularly challenging in cases where novel poly-

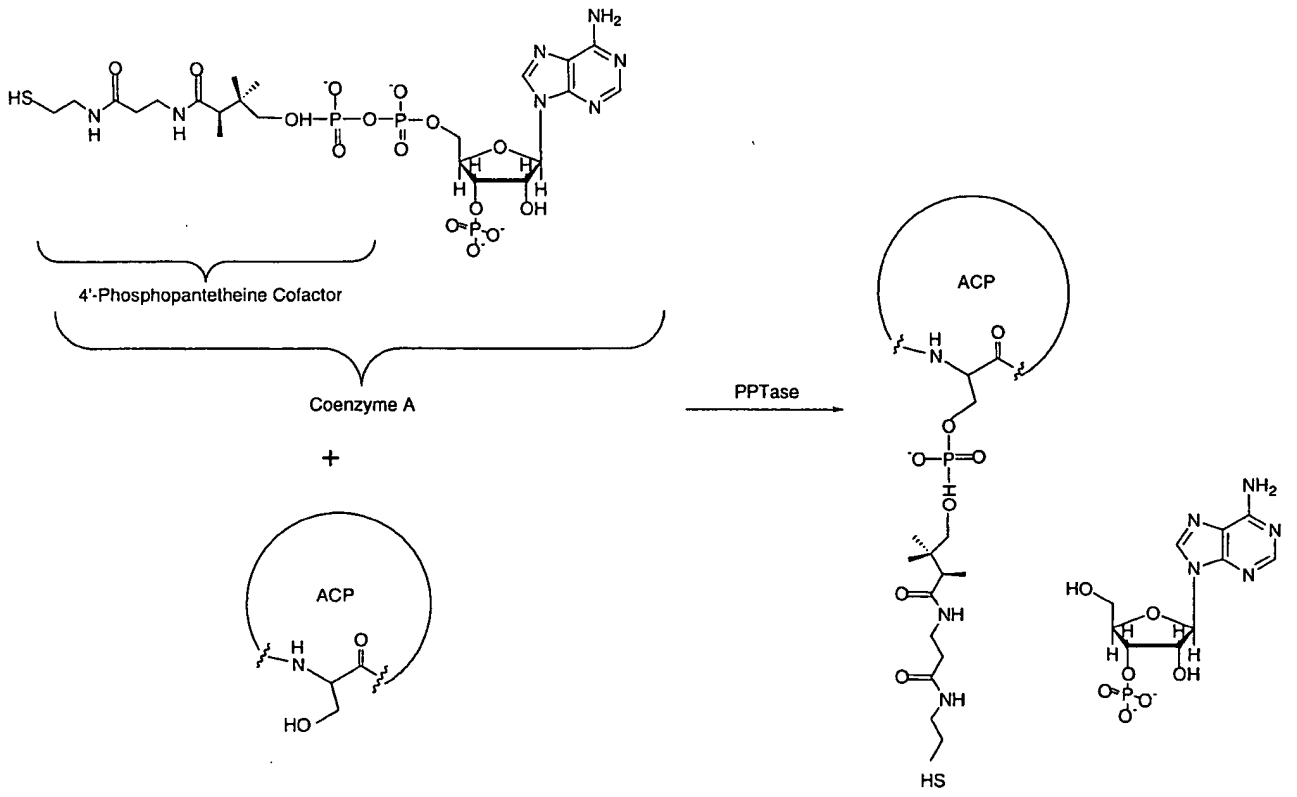

FIG. 7. PPTase-catalyzed reaction. The PPTases catalyze the transfer of the 4'-phosphopantetheine arm from free CoASH to a conserved ACP serine. This "swinging arm" facilitates polyketide transfer during biosynthesis.

ketides are engineered for which no known resistance mechanisms have been identified.

CANDIDATE HOSTS FOR HETEROLOGOUS POLYKETIDE PRODUCTION

The actual choice of a candidate heterologous host depends on the objective of the exercise. Three general reasons might motivate the transfer of a polyketide pathway from a natural source into a heterologous host. A possible goal could be overproduction of the target natural product. Many useful polyketides are derived from sources that are virtually impossible to ferment on a large scale (e.g., a marine sponge or dinoflagellate, where production is most probably encoded by a symbiotic microbe) or are produced in small quantities by a microbe with unsatisfactory growth characteristics. In such scenarios, successful transfer of the biosynthetic capability into a genetically and physiologically characterized heterologous host can provide an attractive alternative starting point for subsequent strain and process development. A second reason for heterologous expression might be to provide a more efficient platform for combinatorial biosynthesis. Many wild bacterial and fungal strains that produce natural products represent extremely challenging targets for genetic manipulation or biochemical analysis. This is in sharp contrast to the plethora of genetic tools and physiological insights that are available for model organisms such as those described below. Finally, the lateral transfer of polyketide biosynthesis genes might be motivated by the phenotypic implications of synthesizing a bioactive compound (or a library of its derivatives) in a heterologous host. For example, the production of a clonal library of small-molecule ligands and their target receptor within the same cell could facilitate the design of a selectable system for ligand optimization. Likewise, the production of an antifungal or insecticidal agent in a plant of agronomic significance could herald a new paradigm for crop protection. Below, we highlight the characteristics of selected heterologous hosts that make them well suited for polyketide production.

Streptomyces coelicolor

For many reasons, *S. coelicolor* is an ideal host for the heterologous production of polyketides. As the first few genes encoding polyketide biosynthesis were identified (3, 5, 13, 17, 21, 78), a suitable host was desired for expressing these genes (in either wild-type or altered forms). The extensive knowledge about the biology of *S. coelicolor*, coupled with its membership of the *Streptomyces* genus, made this host a desirable choice. *S. coelicolor* naturally produces at least two known polyketides of its own (Fig. 2), actinorhodin (50) and the *whiE* spore pigment (14), although the latter is exclusively spore associated and is not produced in liquid culture. To eliminate background polyketide "noise" due to the actinorhodin pathway, a genetically engineered "clean" host strain, CH999, was constructed,

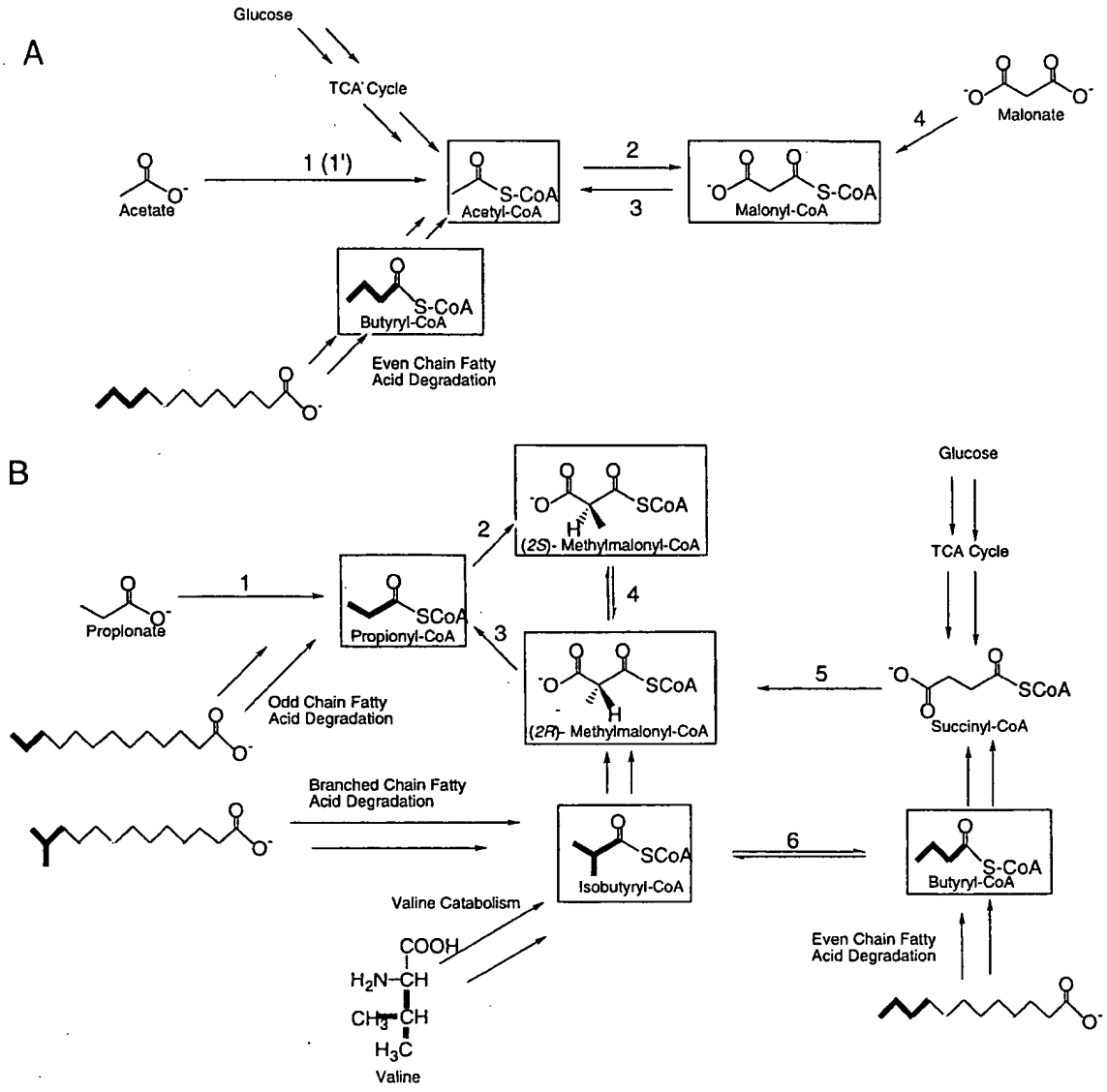

FIG. 8. Polyketide synthase substrate routes. Potential substrates are boxed. (A) Enzymes performing one enzymatic conversion: 1, acetyl-CoA synthetase (alternatively, 1′ represents a two-enzyme pathway, acetate kinase followed by acetylphosphotransferase); 2, acetyl-CoA carboxylase; 3, malonyl-CoA decarboxylase; 4, malonyl-CoA synthetase. (B) Enzymes performing one enzymatic conversion: 1, propionyl-CoA synthetase (1′, propionate kinase followed by propionylphosphotransferase); 2, propionyl-CoA carboxylase; 3, methylmalonyl-CoA decarboxylase; 4, methylmalonyl-CoA epimerase; 5, methylmalonyl-CoA mutase; 6, isobutyryl-CoA mutase.

in which the entire actinorhodin (*act*) gene cluster was surgically deleted via homologous recombination and replaced with an *ermE* marker gene (56). Concomitantly, a low-copy shuttle vector, pRM5, was engineered, which carries the *actI-actIII* bidirectional promoter together with the *actII*-ORF4 gene, which encodes an activator for the *actI-actIII* promoter (56). Introduction of heterologous PKS genes under the control of this expression system allows the production of polyketides in a secondary metabolite-like manner in a wide range of actinomycete strains including *S. coelicolor* (56), *S. lividans* (88), *S. parvulus* (43), and *S. erythraea* (72). The list of bacterial and fungal polyketides produced using this host-vector system includes products derived from the frenolicin (57), tetracenomycin (56), oxytetracycline (23), R1128 (53), erythromycin (39), picromycin/methymycin (81), oleandomycin (77), megalomicin (84), 6-methylsalicylic acid (4), and epothilone (82) gene clusters. (In some cases, for technical convenience, a "clean" derivative *of S. lividans*, K4-114, which also lacks the native *act* gene cluster, has been used as a heterologous host [88].) PKS proteins are produced at ~1% of the total cellular protein levels (67), and the titers of the resulting polyketide products are typically in the range of 1 to 100 mg/liter of culture.

The introduction of polyketide pathways into *S. coelicolor* has also influenced the ease with which PKSs can be genetically engineered for the production of "unnatural" natural products. Type II and modular PKSs have been particularly fertile tar-

gets of manipulation in this regard. Libraries of novel aromatic polyketides (58, 86) as well as macrolides (59) have been generated by the combinatorial manipulation of PKS domains and subunits. In turn, this has led to the emergence of heuristics for regioselective modification of polyketide structures. While considerable work lies ahead to increase the predictability of these heuristics to the level of the "codon table" for polypeptide biosynthesis, it is reasonable to anticipate that this enhanced biosynthetic capability will draw heavily from advances in heterologous expression technologies for PKS gene clusters.

A principal limitation of *S. coelicolor* is that its polyketide natural products (actinorhodin and the spore pigment) are exclusively synthesized from malonyl-CoA-derived building blocks. As such, its metabolic apparatus appears to be limited in the supply of other PKS substrates, such as methylmalonyl-CoA, and the productivity of heterologous polyketides derived from these substrates may suffer. Efforts are under way to better understand the pathways employed by *S. coelicolor* for PKS substrates. For example, genome-sequencing efforts have already led to the identification of its putative methylmalonyl-CoA mutase subunits (GenBank accession number AL138668); understanding the mechanisms by which the activity of this enzyme is regulated at both the transcriptional and posttranscriptional levels could lead to more efficient conversion of glucose into polyketides via succinyl-CoA (a key tricarboxylic acid pathway intermediate). Likewise, at least three different acyl-CoA carboxylase enzymes are encoded within the *S. coelicolor* genome (71). This apparent redundancy in the biosynthesis of α-carboxylated CoA thioesters might suggest that different alleles have different substrate preferences (e.g., for acetyl-CoA versus propionyl-CoA) and/or regulatory features in response to the transition from primary metabolism (when only malonyl-CoA derived fatty acids are synthesized) to secondary metabolism (when polyketides are synthesized). Finally, the potential exists for the incorporation of entirely new pathways devoted to substrate production. For example, heterologous expression of the genes encoding the malonyl-CoA synthetase and dicarboxylate transporter protein from *Rhizobium trifolii* into *S. coelicolor* led to a substantial enhancement in the yield and productivity of methylmalonyl-CoA-derived erythronolide (unpublished data). Importantly, since this synthetase can activate a wide range of 1,3-dicarboxylic acids into the corresponding CoA thioesters (N. L. Pohl, Y. S. Kim, and C. Khosla, submitted for publication), it provides a potentially useful route for the intracellular generation of substrates that are ordinarily unavailable for polyketide biosynthesis.

In addition to optimizing cellular metabolism in *S. coelicolor*, further developments in understanding and maximizing gene expression (especially in response to the onset of secondary metabolism) are expected to have a significant impact on the utility of this host for the production of heterologous PKSs and polyketides. Of the different promoters that have been investigated thus far (which include the *actI* [21], *tipA* [62], and *ermE* [6] promoters), the *actI* promoter appears to have the most favorable characteristics with respect to both expression levels and induction at the onset of the stationary phase. However, the exact signals that lead to maximal induction of this promoter system have not yet been elucidated (54), and PKS proteins are produced only during a relatively short (ca. 24-h) window. Moreover, utilization of this expression system on high-copy-number vectors can lead to instability for reasons that are not understood, and the development of vectors that span a range of copy numbers could be valuable in this regard. Together, these features make controlled, high-level expression of PKS genes in *S. coelicolor* a challenging yet opportune problem.

Escherichia coli

In general, the utility of *E. coli* as a host for heterologous gene expression as well as metabolic engineering is unquestioned. However, in order to reconstitute polyketide biosynthesis in *E. coli*, several issues beyond gene expression needed to be addressed. Unlike *S. coelicolor, E. coli* is a significantly different heterologous host from those that naturally produce polyketide products. The robust *E. coli* expression systems available can induce PKS proteins to accumulate as inclusion bodies; however, the judicious control of temperature, medium composition, and other induction conditions yields substantial levels (1 to 5% of total cellular protein) of correctly folded protein for PKSs with molecular masses of >200 kDa (unpublished data). Likewise, coexpression of the *sfp* PPTase gene in a plasmid-borne or chromosomal format leads to stoichiometric pantetheinylation of soluble PKS proteins in *E. coli* (B. Pfeifer, unpublished results). Also, the high G+C content of actinomycete PKS genes leads to an inappropriate bias in codon usage that is difficult to remedy using synthetic gene approaches due to the large ORF sizes. The use of host strains that contain extra copies of the rare AGG (arginine) and CCC (proline) codons can be helpful in this regard (reference 74 and unpublished data). The availability of PKS substrates in *E. coli* presents another major challenge, since biosynthesis of malonyl-CoA is under tight control (40) and substrates such as propionyl-CoA and methylmalonyl-CoA are produced under poorly understood conditions (34). Reconstitution of heterologous substrate generation pathways in this host has provided a useful starting point for addressing this problem (unpublished data). Finally, the availability of well-established protocols for two-stage, high-cell-density fermentation protocols, where the growth phase can be decoupled from PKS gene expression and polyketide production, could provide rapid development of processes that yield high volumetric productivities for both PKSs and polyketides (46).

A variety of PKSs have been functionally expressed in *E. coli*. For example, coexpression of the *sfp* and 6-methylsalicylic acid synthase genes in *E. coli* was found to be necessary and sufficient for the intracellular production of 6-methylsalicylic acid, although polyketide production was substantially enhanced under conditions that ordinarily favor higher rates of malonyl-CoA formation (40). *E. coli* has the means to produce acetyl-, malonyl-, propionyl-, and possibly even methylmalonyl-CoA, and one might expect the ability to increase these levels based on pathway alteration. However, other options also exist. For example, a pathway leading to the formation of malonyl- and (2*S*)-methylmalonyl-CoA PKS substrates, which has been successfully reconstituted in *E. coli*, is based on the *S. coelicolor* acetyl-CoA carboxylase and propionyl-CoA carboxylase enzyme complexes that use biotin as a cofactor (unpublished data). The genes for acetyl-CoA carboxylase and propionyl-CoA carboxylase have recently been cloned and overexpressed

in *E. coli* in an active form and have shown the ability to provide substrates for polyketide formation within *E. coli* (71). In summary, although *E. coli* presents a completely new environment for PKS expression and polyketide formation, it represents a promising opportunity for harnessing the biosynthetic capabilities of polyketide pathways. The ability to produce 6-deoxyerythronolide B in *E. coli* in a robust manner is an example of realizing this promise (unpublished data).

Other Actinomycetes

For reasons similar to those that prompted the utilization of *S. coelicolor* as a host for polyketide biosynthesis, other polyketide-producing actinomycetes could be considered. For example, as mentioned above, *S. lividans* has been successfully used to express several PKS systems, due to its close relationship to *S. coelicolor* and its greater transformation efficiency (which, for example, readily allows the introduction of multiple plasmids [85]). Similarly, *S. glaucescens* (76) and *Saccharopolyspora erythraea* (27) have also been tested as heterologous hosts for polyketide production. These hosts have naturally occurring polyketide biosynthetic pathways and may therefore be better suited for the expression of heterologous PKS genes. Finally, of particular interest in the development of heterologous hosts would be the potential utility of highly evolved polyketide-producing strains. For example, through multiple cycles of random mutagenesis and screening, derivatives of *Saccharopolyspora erythraea* have been selected that produce 8 g erythromycin per liter (which corresponds to a titer that is 50 to 100 times that in the wild-type strain) (60). Recent studies have demonstrated that the genetic basis for overproduction in such hosts is not encoded within the PKS genes themselves but within other loci on the genome (R. McDaniel, unpublished results). Given the multigenic nature of this trait, the development of such organisms as hosts for polyketide production may present an attractive option. Moreover, a systematic dissection of the genetic and physiological basis for overproduction could yield insights that might readily be translated to naive hosts such as *S. coelicolor* and *E. coli*.

Other Bacteria

Although the actinomycetes are perhaps the most prolific producers of polyketides, several other bacterial families are known to synthesize structurally diverse polyketides. Chief among these are the myxobacteria, pseudomonads, and mycobacteria. For example, myxobacteria such as *Sorangium* spp. produce polyketides including soraphen (31) and epothilone (30). Among other compounds, *Pseudomonas* spp. produce pseudomonic acid (51) and coronatine (70). *Mycobacterium ulcerans* produces the highly potent immunosuppressive agent mycolactone (29), whereas the genome of *Mycobacterium tuberculosis* appears to be well endowed with a plethora of as yet uncharacterized PKS pathways (12). Although genetically well-characterized strains representative of these bacterial families, such as *Myxococcus xanthus, Pseudomonas putida*, and *Mycobacterium smegmatis*, are known, the credentials of these bacteria as hosts for heterologous polyketide production have not yet been seriously evaluated.

Fungi

Filamentous fungi too are prolific producers of polyketides. The utility of the fungus *Aspergillus nidulans* as a heterologous host has been successfully demonstrated in the context of lovastatin biosynthesis (41). A major advantage of such hosts would be the ability to splice introns that are frequently present among eukaryotic PKS genes. Moreover, given the well-established fermentation processes for lovastatin and compactin biosynthesis, it is reasonable to assume that highly evolved strains capable of overproducing these metabolites may be available. If so, these systems could present attractive options for reverse engineering into generic hosts for polyketide biosynthesis. Finally, given the premiere status of the yeast *Saccharomyces cerevisiae* in fungal and eukaryotic genetics and its designation as a GRAS host (Generally Regarded as Safe), its use as a host for polyketide production ought to be seriously investigated. Although yeast has no known polyketide biosynthetic pathway encoded within its genome, it does have a highly active fatty acid biosynthetic pathway. Moreover, recent studies have shown that it is capable of synthesizing polyketides at high levels (40), although several features must be introduced into the yeast genome before this can be generalized.

Plants

Plants offer another option as potential hosts for the heterologous production of polyketides. They are known to produce a number of polyketide products (e.g., chalcones, stilbenes, and coumarins), and their chloroplasts provide a "bacterium-like" environment that appears to be particularly well suited for fatty acid biosynthesis. Over the past two decades, major advances in plant genetic engineering have led to their emergence as viable hosts for heterologous expression and metabolic engineering. In particular, the ability to produce complex polyketides in plants could have a significant impact on strategies for crop protection in transgenic plants.

FUTURE DIRECTIONS

A critical choice in heterologous expression of any protein is the host; this is especially the case when multiple proteins must be coexpressed to effect a phenotypic alteration that is often the target of metabolic engineering. In addition to laboratory convenience, the chosen heterologous hosts must possess (or be engineered to possess) the cellular machinery required for successful protein production and sustained activity. Following in the footsteps of recombinant polypeptide biosynthesis, recombinant polyketide biosynthesis presents a major challenge as well as an opportunity for molecular biology. This article attempts to highlight the unique challenges encountered during the course of heterologous polyketide production. The current state of the art is presented both with respect to PKS gene expression and metabolic engineering. We speculate that, as has occurred with polypeptides, over the next decade a few heterologous systems will emerge as workhorse tools for exploiting the enormous chemical diversity of naturally occurring polyketides. Key criteria that will influence this decision will be the case of manipulating PKS genes in these hosts, metabolic robustness, volumetric productivity (grams of the polyketide

produced per liter per hours), and generality of use with different PKS pathways. However, as it becomes increasingly simpler to introduce polyketide pathways into new biological systems, the biologist will be empowered with a unique set of small-molecule-based tools to probe cellular structure and function. This, in turn, should fuel further research into strategies for lateral transfer of polyketides across taxonomic boundaries. After all, this is precisely what nature appears to have accomplished in its attempt to maximize the evolutionary utility of this remarkable library of functional biomolecules.

ACKNOWLEDGMENTS

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Production of Hybrid 16-Membered Macrolides by Expressing Combinations of Polyketide Synthase Genes in Engineered *Streptomyces fradiae* Hosts

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Summary

Combinations of the five polyketide synthase (PKS) genes for biosynthesis of tylosin in *Streptomyces fradiae* (*tylG*), spiramycin in *Streptomyces ambofaciens* (*srmG*), or chalcomycin in *Streptomyces bikiniensis* (*chmG*) were expressed in engineered hosts derived from a tylosin-producing strain of *S. fradiae*. Surprisingly efficient synthesis of compounds predicted from the expressed hybrid PKS was obtained. The post-PKS tailoring enzymes of tylosin biosynthesis acted efficiently on the hybrid intermediates with the exception of TylH-catalyzed hydroxylation of the methyl group at C14, which was efficient if C4 bore a methyl group, but inefficient if a methoxyl was present. Moreover, for some compounds, oxidation of the C6 ethyl side chain to an unprecedented carboxylic acid was observed. By also expressing *chmH*, a homolog of *tylH* from the chalcomycin gene cluster, efficient hydroxylation of the 14-methyl group was restored.

Introduction

The 16-membered macrolides (16-MM) are an important class of antibiotic that are used in human and veterinary medicine. They are structurally related to the better-known 14-membered macrolides (see Figure 1). Biosynthesis begins with assembly of a macrolactone ring by a modular polyketide synthase (PKS) [1]. Six different 16-membered macrolactone ring structures are known, from which more than 200 antibiotics are derived through different post-PKS tailoring reactions [2]. The PKSs for all known 16-MMs consist of five large polypeptides with a conserved modular organization. The macrolactone product is determined by the specificities of catalytic domains within each module. Figure 2 shows the domain organization for the tylosin (*tyl*), spiramycin (*srm*), and chalcomycin (*chm*) PKSs, as well as the macrolactone product of each enzyme, tylactone, platenolide, and chalcolactone, respectively. Both the biosynthesis of tylosin and the involvement of genes in the tylosin cluster have been studied extensively [3–14]. Less has been reported on spiramycin biosynthesis, although the PKS genes were cloned and sequenced some time ago [15, 16]. We have recently characterized the chalcomycin gene cluster [17] and found some unusual features with respect to formation of the 2,3-*trans* double bond.

This work focused on engineering the biosynthesis of 16-MMs related to midecamycin A1 (see Figure 1) to introduce a chemical handle for attachment of side chains on the left side of the molecule that could potentially enhance antibiotic activity against macrolide-resistant strains. Three macrolide binding regions of the 50S ribosomal subunit are associated with antibacterial activity (1) near the residue corresponding to adenosine 2058 of the 23S rRNA (*Escherichia coli* numbering) in domain V, (2) in the peptidyl transferase active site of domain V, and (3) in domain II (hairpin 35) of the 23S rRNA [18–26]. All macrolides interact with the A2058 region and methylation of this residue substantially reduces binding and confers high-level resistance [22, 23, 27]. Midecamycin A1 and other 16-MMs that have an acyl group extending from the disaccharide component can reach into the peptidyl transferase site and inhibit its activity [20, 21], providing some restoration of potency against resistant strains [28]. Ketolides such as telithromycin, derived from erythromycin by attachment of a side chain, are active against some macrolide-resistant pathogens such as *Streptococcus pneumoniae* [29]. The side chain interacts with nucleotides in the domain II region [26, 29]. Most 16-MMs are not amenable to chemical attachment of side groups for domain II binding, and the PKS engineering described here was aimed at installing such a handle. Specifically, we engineered hybrid 16-MMs with a 14-hydroxymethyl group that should allow attachment of side chains via cyclic carbamates between the 14-hydroxymethyl group and an unhindered 12,13 double bond, similar to chemistry used in the synthesis of telithromycin [29].

To produce 16-MMs derived from hybrid macrolactones consisting of chalcolactone and either platenolide or tylactone, the first two genes of the *chm* PKS cluster, encoding the loading and three extender modules, were expressed along with the last three from either the *srm* or *tyl* PKS, encoding four extender modules, in specially constructed *S. fradiae* hosts in which the native *tyl* PKS-encoding genes were either deleted or inactivated. In these strains, all or most of the *tyl* biosynthesis genes involved in post-PKS processing were present. Introduction of hybrid PKSs into these hosts resulted in efficient production of novel structures derived from the predicted macrolactone. However, some of these molecules were not efficiently hydroxylated on the 14-methyl group by the TylH hydroxylase. To correct this, a homologous hydroxylase gene from the chalcomycin producer was introduced.

Results and Discussion

Expression of a Hybrid Chalcomycin-Spiramycin PKS Operon in *S. fradiae* Yields a Series of Novel Compounds

A pSET152-derived vector carrying the *chmGI-II-srmGIII-V* genes as a single operon was introduced into

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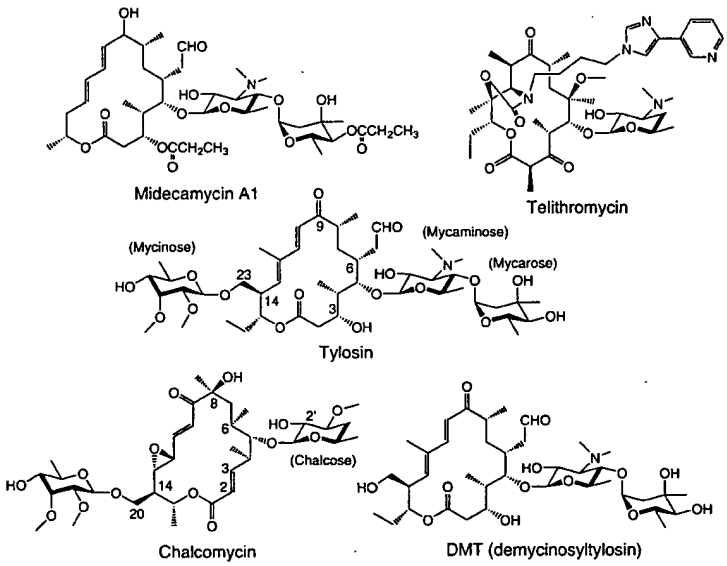

Figure 1. Structures of Macrolide Antibiotics and Intermediates Relevant to the Development of New Antibiotics Using PKS Engineering

S. fradiae K159-1/pKOS244-017a, in which the tylosin PKS genes had been deleted [30], and in which the genes *fkbGHIJK* for the methoxymalonyl-ACP precursor from *Streptomyces hygroscopicus* had been integrated at the pSAM2 *att* site [31]. The methoxymalonyl-ACP precursor is a required substrate for platenolide PKS module 5 [31]. The hybrid PKS operon was expressed from *tylGIp*, which was previously shown to be a strong promoter in *S. fradiae* [30]. To facilitate construction of the hybrid operon, the C-terminal interaction domain [32] of the second chalcomycin PKS polypeptide (ChmGII) was replaced with that of the spiramycin PKS (SrmGII) at a unique HindIII site. Transconjugants were patched on R5 agar, and plugs were screened for bioactivity on *Micrococcus luteus*. After 7 days growth, most plugs gave large zones of inhibition. LC-MS analysis of an ethyl acetate extract of the agar under these zones showed a major peak with a molecular mass of *m/z* 714.85 $[MH]^+$. The methanol adduct was also observed, which is generally diagnostic of an aldehyde function. Upon purification, it was identified by NMR spectrometry as 4″-despropionyl-14-methylniddamycin (DPMN, Figure 3), a compound derived from 14-methylplatenolide. After shake flask fermentation of one of the isolates (*S. fradiae* K232-192), LC-MS analysis of whole broth revealed three major compounds: *m/z* 585.68 $[MH]^+$, *m/z* 714.85 $[MH]^+$, and *m/z* 730.85 $[MH]^+$, in order of abundance, as well as a low level of a compound of *m/z* 905.05 $[MH]^+$. Surprisingly, only DPMN and the *m/z* 905.05 $[MH]^+$ compound were extracted into ethyl acetate, so solid-phase extraction of the spent aqueous phase was used to recover and purify the *m/z* 585.68 $[MH]^+$ compound. NMR analysis revealed its structure to be that of the acidic compound, 6-carboxymethyl-6-desethyl-5-*O*-mycaminosyl-14-methylplatenolide (CDMP, Figure 3), in which the C6 ethylaldehyde had

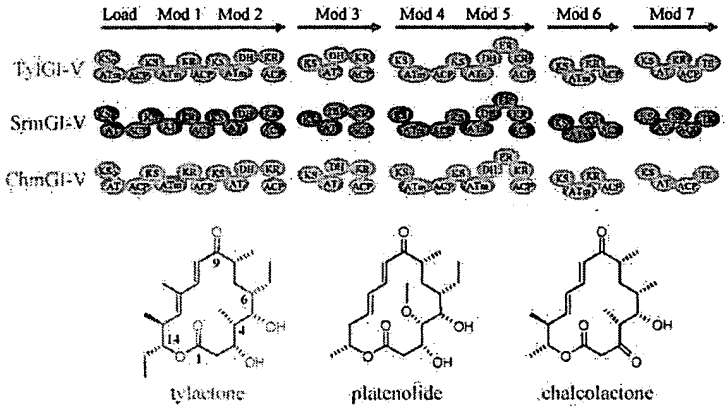

Figure 2. Comparison of the Domain Structure for the Tylosin, Spiramycin, and Chalcomycin PKSs and the Macrolactone Produced by Each

At the top is shown the organization of seven modules on five polypeptides, which is universally conserved among the 16-MMs. Below that the organization of domains within each module is shown for the three PKSs: starter module decarboxylation (KSQ); acyl transferase with specificity for malonyl, methylmalonyl, ethylmalonyl, or methoxymalonyl extender units (AT, ATm, ATe, and ATx, respectively); acyl carrier protein (ACP); ketosynthase (KS); ketoreductase (KR); dehydratase (DH); enoylreductase (ER); and thioesterase macrolactonization (TE). The structures of the PKS products are shown at the bottom with chalcolactone being the structure obtained by expression of the chalcomycin PKS in *S. fradiae* and not necessarily the structure obtained from the PKS in its native context in *S. bikiniensis* [17].

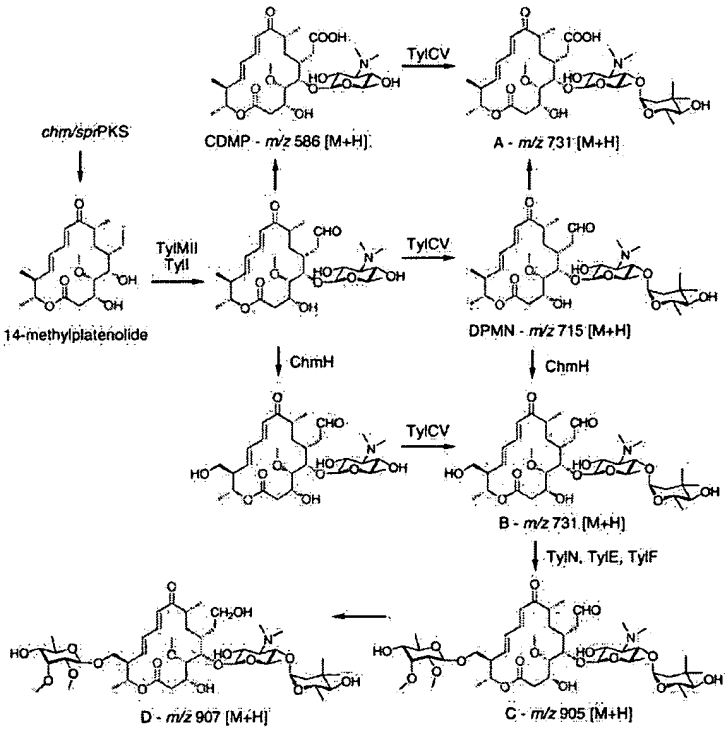

Figure 3. Proposed Post-PKS Tailoring Pathways for the Chalcolactone-Platenolide Hybrid

The portions of the polyketide core derived from the corresponding PKS are indicated using the same color coding as in Figure 2.

been oxidized to the corresponding carboxylic acid, a feature not previously reported for a 16-MM. It was inferred that the *m/z* 730.85 $[MH]^+$ compound was 4'-O-mycarosyl-CDMP, i.e., it also had the carboxylic acid (labeled A in Figure 3). The low level of hydroxylation of the C14 methyl group indicates that intermediates derived from the chalcolactone-platenolide hybrid structure are poor substrates for the TylH hydroxylase of the host. Oxidation of the aldehyde to the carboxylic acid may be catalyzed by TylI, which is known to generate the aldehyde through two sequential hydroxylations, or by an unlinked P450 enzyme that happens to recognize the intermediate as a substrate.

Estimation of titers from areas under the peaks at 280 nm compared with a standard curve of purified DPMN, indicated the total polyketide titer was only slightly less than that of tylosin produced by the parent strain under the same fermentation conditions (~2 g/l). Previously, the only report of a hybrid PKS composed exclusively of modules from 16-MM PKSs was the spiramycin PKS in which the loading domain was exchanged with the loading domain from the tylosin PKS, resulting in production of 15-methylspiramycin [16]. Although an engineered PKS often has reduced catalytic efficiency, either because of impaired PKS function through structural distortion or because the unnatural intermediate is a poor substrate for a downstream step, production of polyketides by this hybrid 16-MM PKS was nearly as efficient as tylosin production by the parent strain. The functional compatibility between different 16-MM PKS polypeptides presumably reflects the significant sequence similarity resulting from relatively recent divergence from a common ancestor. Although replacing the C-terminal interaction domain of ChmGII may not have been necessary, since these domains are highly conserved among the 16-MM PKSs, it was clearly not detrimental.

Expression of *chmH* in *S. fradiae* K232-192 Enhances 14-Methyl Hydroxylation

The chalcomycin gene cluster encodes a close homolog of the TylH hydroxylase, designated ChmH [17]. Each has a ferredoxin gene immediately downstream that may be important for activity or specificity. To determine whether the ChmH hydroxylase would accept a chalcolactone-platenolide derivative as a substrate for hydroxylation of the C14 methyl group, the *chmH* gene with its cognate ferredoxin gene was introduced into the host expressing the *chmGI-II-srmGIII-V* hybrid PKS genes using a modified version of the ϕBT1-based integrating vector, pRT802 [33], carrying *tsr* for thiostrepton resistance and *tylGIp* for expression of inserted genes. Introduction of *chmH* had a dramatic affect on the profile of compounds produced by the strain (Figure 4). Although there was still a significant amount of CDMP present, compounds with masses of *m/z* $[MH]^+$ 730.85, 907.04, and a trace of 905.04 were also observed. Since these three compounds could be extracted into ethyl acetate (Figure 4C), they are inferred to be the compounds labeled B, C, and D, respectively, in Figure 3. Although only the structures of DPMN and CDMP were verified by NMR spectrometry, the structures of the other compounds can be inferred from the well-characterized post-PKS tylosin pathway, the ethyl acetate extractability of the compounds, the presence or absence of a methanol adduct (hemiacetal), and the LC-MS data itself

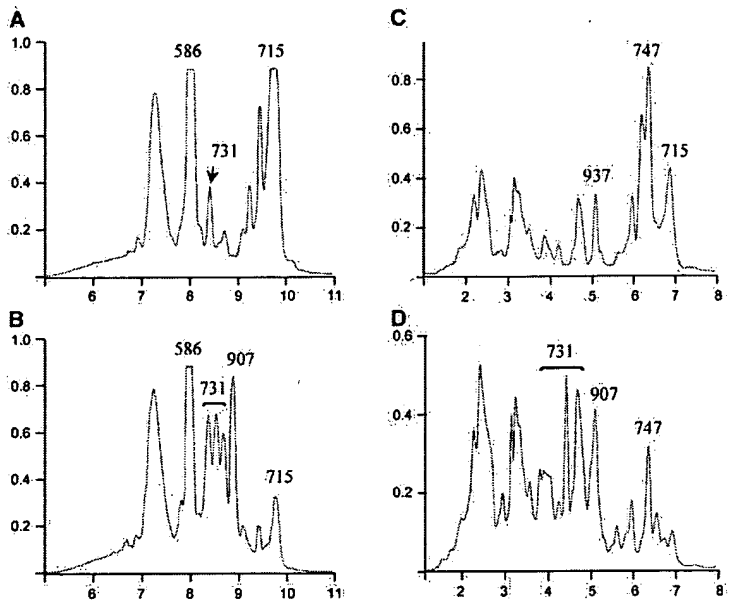

Figure 4. Effect on the Profile of Products Derived from Fermentation of the Strain Expressing the Chalcolactone-Platenolide Hybrid PKS when the *chmH* Gene Is Also Expressed

Shown are the LC-MS traces at 280 nm with the masses of specific peaks of interest indicated above. The x axis indicates the time in minutes from sample injection and the y axis indicates the absorbance at 280 nm. (A) and (B) show products from the strain not containing the *chmH* gene, while (C) and (D) show products from the strain that did contain this gene. (A) and (C) show the LC-MS traces for clarified whole fermentation broth, while (B) and (D) show the traces for products extracted from whole broth into ethyl acetate and the dried residue dissolved in methanol.

(relative retention times and masses). Thus, ChmH can efficiently hydroxylate the 14-methyl group of the intermediates derived from the *chm-srm* hybrid PKS, even though TylH does so very poorly. Figure 3 presents the post-PKS tailoring pathways for the chalcolactone-platenolide hybrid both in the presence or absence of ChmH. It appears that once hydroxylation of the 14-methyl group occurs, the resulting intermediates are resistant to oxidation of the aldehyde.

The first two post-PKS reactions of tylactone are attachment of mycaminose to the C5 hydroxyl by TylMII and oxidation of the C6 ethyl side chain to an aldehyde through two sequential hydroxylations by TylI [4, 6]. All known 16-membered macrolactones have a sugar at the C5 hydroxyl, usually mycaminose, although chalcomycin has chalcose instead [34]. When the macrolactone bears a C6 ethyl side chain, it is always oxidized to an aldehyde. The enzymes for these first two reactions in the tylosin pathway apparently have considerable substrate tolerance, since all the compounds observed here had at least these two post-PKS modifications. Although there is a preferred pathway for the post-PKS reactions in tylosin biosynthesis [4], the order of some steps is flexible and depends on the relative substrate tolerance of the enzymes. For example, a *tylD* mutant of *S. fradiae* produces demycinosyltylosin (DMT) [6], indicating that attachment of the allose sugar does not have to precede attachment of the mycarose sugar.

The significant level of the *m/z* 907 $[MH]^+$ compound produced is believed to result from reduction of the aldehyde of the *m/z* 905 $[MH]^+$ compound to the alcohol. This reaction converting tylosin to tylosin D is known to occur in *S. fradiae*, and the enzyme responsible has been characterized [35]. Apparently, the tylosin analog derived from expression of the *chmGI-II-srmGIII-V* hybrid PKS is a particularly good substrate for this enzyme. Strain K232-192 not expressing *chmH* did not produce detectable *m/z* 907 $[MH]^+$ compound, though it did produce a low level of the *m/z* 905 $[MH]^+$ compound. Perhaps a threshold level of the *m/z* 905 $[MH]^+$ compound is required to induce the reductase activity.

Complementation of a KS1° *S. fradiae* Strain with the First Two Genes of the Chalcomycin PKS Gives a Single Novel Compound

Changing the active site cysteine residue to an alanine in the first keto synthase (KS) domain of the erythromycin PKS blocks synthesis of the product, 6-deoxyerythronolide B, but allows appropriate diketides, as their N-acetylcysteamine thioester derivatives, to be fed to the KS1° host, giving the corresponding macrolactones and showing that modules 2–6 of the PKS are functional in the mutant [36]. When a similar mutation was introduced into the KS1 domain of the *tyl* PKS of a DMT producer of *S. fradiae*, i.e., bearing a *tylD* mutation [5], feeding of the appropriate diketide thioester also restored production of DMT (data not shown), indicating that modules 2–7 in the *tyl* PKS were functional. A synthetic operon containing the *chmGI-II* genes under the control of *tylGIp* were integrated into the chromosome of this *S. fradiae* strain at the ϕC31 *att* site using a derivative of pSET-152 [30]. Although the heterologous ChmGII polypeptide (carrying module 3) can probably interact appropriately with the host TylGIII polypeptide (carrying modules 4 and 5), the C-terminal interaction domain of ChmGII was replaced with the TylGII counterpart to ensure an optimum interaction based on the results with the ChmGI-II-SrmGIII-V hybrid discussed above. The resulting strain (*S. fradiae* KS1°/pKOS342-84) produced antibiotic activity against *M. luteus*, and LC-MS analysis of the fermentation broths revealed a compound of *m/z* 714.88 $[MH]^+$. Isolation and NMR analysis showed the structure to be 12,16-didesmethyl-DMT, in agreement with the prediction. The titer of 12,16-didesmethyl-DMT was approximately 0.5 g/l, indicating that the presence of the inactive TylGI and active TylGII

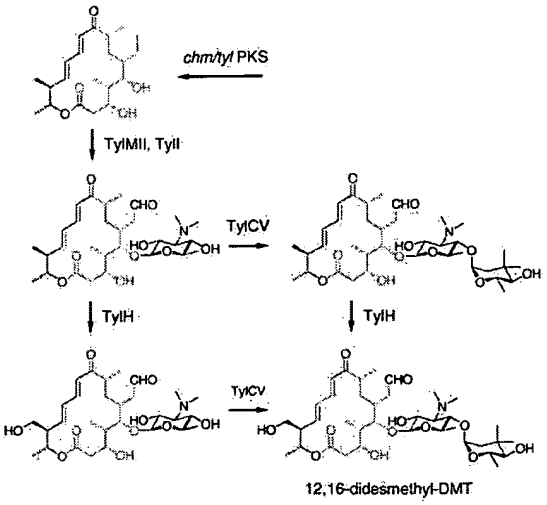

Figure 5. Proposed Post-PKS Tailoring Pathways for the Chalcolactone-Tylactone Hybrid in the *tylD*, KS1° Double Mutant Strain
See text for details.

polypeptides did not significantly interfere with the interaction between ChmGI and either ChmGII or TylGII, which have identical domain organization and should both interact with TylGIII.

Figure 5 shows the post-PKS tailoring pathways leading to the chalcolactone-tylactone hybrid. Since 12,16-didesmethyl-DMT was produced efficiently, the presence or absence of methyl groups on C12 and C16 has little effect on TylH-catalyzed hydroxylation of the 14-methyl group (or the other post-PKS reactions). Although the 14-methyl group of the *chmGI-II-tylGIII-V* hybrid PKS product is efficiently hydroxylated by TylH, neither the product of the *chmGI-II-srmGIII-V* hybrid PKS nor the product of the complete chalcomycin PKS [17] are good substrates for 14-methyl hydroxylation by TylH. This suggests that an oxygen atom on C3 or C4 with a particular position relative to the 14-methyl group may inhibit the TylH hydroxylase. Because the ChmH hydroxylase presumably evolved to function in the presence of this oxygen, it is perhaps not surprising that it can hydroxylate the chalcolactone-platenolide hybrid.

Significance

An important goal of PKS engineering is to generate unique molecules that can be used as starting points for subsequent chemical modification. The 14-hydroxymethyl derivatives produced in this study are examples of potential starting points. These compounds lack the 12-methyl side chain of tylosin and its intermediates and should allow cyclization chemistry on the left side of the molecules equivalent to that employed in ketolide synthesis in the 14-membered macrolide group. It has also been observed previously that the catalytic efficiency of hybrid or mutant PKS enzymes can be significantly reduced. This work shows that coexpression of genes encoding the polypeptides of different, but related, PKSs can give highly efficient production of novel products. The strategy is useful in cases where there are several highly homologous sets of PKS genes available that encode the production of slightly different structures. Thus, use of the *S. fradiae* host strains described here for expression of PKS gene combinations allows production of 16-MMs derived from novel macrolactones. The post-PKS tylosin pathway is generally tolerant of structural variations, although the TylH hydroxylase could not hydroxylate the 14-methyl group of one of the hybrids. In cases where a post-PKS enzyme of the tylosin pathway will not accept the hybrid structure as a substrate, there may be an alternative enzyme that can be recruited from another 16-MM pathway, as was demonstrated for the ChmH hydroxylase.

Experimental Procedures

Strains, Growth Media, and Basic DNA Manipulation

All strains were derived from a tylosin-overproducing *S. fradiae* strain [30]. Deletion of the tylosin PKS genes from the strain was described previously [30]. DNA was delivered into *S. fradiae* strains by conjugative transfer of a mobilizable plasmid from *E. coli* DH5α harboring the RK2-derived helper plasmid pUB307 [30]. The conjugation protocol was as described [37], except that overnight incubation prior to the antibiotic overlay was at 37°C. Plasmids were propagated in *E. coli* DH5α and were constructed using well-established methods [38]. Antibiotic-resistant exconjugants of *S. fradiae* were streaked for single colonies on AS-1 medium containing the appropriate antibiotics. When exconjugants of a nonproducing host were expected to produce a tylosin-related analog, they were patched onto R5 medium and, after 7 days, plugs were screened for their ability to form zones of inhibition on lawns of *Micrococcus luteus* ATCC 9341 growing on Difco medium 11.

Construction of *S. fradiae* Strains

S. fradiae K264-105.2 contains a deletion of the *tylD* gene and a null mutation in KS1 of *tylGI*. Deletion of *tylD* involved sequential double homologous crossing over after introduction of a plasmid carrying regions flanking the gene. The ca. 1.8 kb regions were isolated by PCR using the following oligonucleotides: 5′-GGCATGC CCAGAACCAGTACCCGGTCACATG-3′, with 5′-GCTGCAGCCCAC TCGTGAATCCCGAAGGGAAG-3′, and 5′-CCTGCAGCGTAGTGGG AGCCGATGAAGCCCA-3′, with 5′-GGAATTCCTGACACAGACCGG TCACCGTTCGT-3′. This introduced the unique restriction sites (EcoRI-PstI and PstI-SphI). The PCR products were ligated between the EcoRI and SphI sites of pOJ260 [39] giving pKOS168-106, in which 80% of the *tylD* gene was removed and the reading frame maintained. After conjugation of the *E. coli* donor with the *S. fradiae* K155-3C [30] recipient, apramycin-resistant colonies were obtained, and those with the plasmid integrated at the *tylD* locus were identified by Southern blot hybridization. Growth in the absence of selection gave *S. fradiae* K168-173, which had become apramycin sensitive and produced DMT. The KS1° mutation was introduced by changing the active site cysteine to alanine and introducing a NheI site. This was accomplished using PCR to make two fragments. The first was a 260 bp fragment made using the oligonucleotides 5′-ATGGATCCGCAGCAGCCCGTGT-3′ and 5′-GCTAGCCGCCGTAT CCACGGTCACGG-3′. The second was a 370 bp fragment made using the oligonucleotides 5′-GCTAGCTCGTCGTTGGTGGCGTTG CATCT-3′ and 5′-GCGCATTCCCCAACGCCTGACGAAT-3′. The first fragment was digested with BamHI and NheI and the second fragment with NheI and BsmI. These two pieces were then ligated into BamHI and BsmI digested pKOS264-65, which is pUC19 containing the ca. 6 kb EcoRV/EcoRI fragment from *tylGI*, to generate pKOS325-8. The insert from pKOS325-8 was isolated by digestion with XbaI and PvuII and ligated into pKOS241-52 (derived from pSET152 by cutting with SphI/HindIII, blunting with Klenow Polymerase, and self ligating). This suicide plasmid, designated pKOS264-76, was introduced into *S. fradiae* K168-173 using an *E. coli* donor.

The first and second crossover events were verified by Southern blot hybridization and the strain, K264-105.2 was shown to produce no tylosin-related compounds. However, it did produce DMT when 2(S)-methyl-3(R)-hydroxypentanoyl-N-acetylcysteamine was fed to the strain (M.M., W.P.R., and L.K., unpublished data).

Construction of the host with the tylosin PKS genes deleted (*S. fradiae* K159-1) is described elsewhere [30]. Construction of strain K159-1/pKOS244-017a by introducing a set of genes from the FK520 (ascomycin) gene cluster of *S. hygroscopicus* ATCC 14891 (*fkbGHIJK*) that provide the precursor methoxymalonyl-ACP is also described elswhere [31].

Construction of Vectors for Expression of PKS Genes

pKOS232-189 is an expression vector similar to pSET152 [39], but containing *tylGIp* with a NdeI site at the start of transcription, and a λ cos site for packaging of large constructs. The *tylGIp* was amplified by PCR [30], ligated into the EcoRV site of Litmus38, then isolated as a NdeI/PstI fragment. This fragment, a 3 kb NdeI/SpeI fragment of heterologous PKS sequence, and pKOS159-31 [30] cut with NsiI and SpeI were ligated together to create pKOS232-189.

The integrative expression vector, pRT802, was made using the *attP* site and *int* genes from the phage φBT1 [33]. Multicloning sites were added using a linker made from the following oligonucleotides: 5′-GGCCGCGTCAATTGGCCGTTAATTAACGCATATGGCCTAGGC GAGGCCTGCATGCATCCAAGCTTCG-3′ and 5′-GATCCGAAGCTT GGATGCATGCAGGCCTCGCCTAGGCCATATGCGTTAATTAACGG CCAATTGACGC-3′, to give pKOS231-149. The *tylGIp* was then introduced by digesting the *tylGIp*/Litmus38 plasmid mentioned above with NotI and NdeI and ligating it into pKOS231-149 digested with the same enzymes to create pKOS231-153D. To isolate the ampicillin resistance gene, Litmus28 was digested with HpaI and SwaI. This was ligated into pKOS231-153D digested with BspHI and end-filled with Klenow polymerase to give pKOS231-183D. To isolate the thiostrepton resistance gene, pIJ5719 (C. Khosla, personal communication) was digested with NdeI, end-filled with Klenow Polymerase, and digested with SmaI. This fragment was ligated to EcoRV-digested Litmus38 to give pKOS342-106B. This was digested with BamHI and HindIII to ligate the thiostrepton resistance gene into pKOS231-183D digested with the same enzymes, which created the expression vector pKOS342-108D.

Constructs for Expression of 16-MM PKS Genes in *S. fradiae*

Characterization of the chalcomycin gene cluster and construction of an expression plasmid containing the first two chalcomycin PKS genes, pKOS232-172, are described elsewhere [17]. To replace the C-terminal interaction domain of ChmGII with the corresponding TylGII linker, a short fragment from the 3′ end of *tylGII* was amplified by PCR using the primers 5′-TGAAGCTTCCCGCCACGCTGGTG-3′, 5′-CGTCTAGACAGGGTGTGAAACCG-3′, and pKOS168-190 [30] as the template, which introduced a HindIII site corresponding to the natural site in *chmGII*. The PCR product was digested with HindIII and XbaI and ligated into pKOS232-172 cut with the same enzymes to create pKOS342-82. The insert of this plasmid was excised with NdeI and XbaI and ligated with the vector portion of pKOS232-189 digested with NdeI and SpeI to give pKOS342-84. This plasmid was moved into *E. coli* DH5α/pUB307 and then into *S. fradiae* K264-105.2 by conjugation to give the strain *S. fradiae* K342-84.

A natural, unique HindIII site near the 3′ end of *chmGII* was used to attach the chalcomycin genes to the last three spiramycin PKS genes. The three spiramycin genes were assembled from subclones of cosmid pKC1306 (unpublished Eli Lilly deposit NRRL B21499). A HindIII site was introduced into *srmGII* at the same position as for *chmGII* by PCR amplification using the following upstream primer: 5′-ACACGCTTAAGACTGAAGCTTCCCGCGACCTCGTCTTC-3′. The other primer was downstream of a natural BamHI site, and thus an 800 bp AflII-BamHI fragment was isolated and ligated between the same sites of pKOS231-114A, which attached the PCR product to most of *srmGIII* down to a BsrGI site (pKOS232-178). The large SpeI to BsrGI fragment of this vector was then ligated to the large BsrGI to AvrII fragment of pKOS231-132, which contained the remainder of *srmGIII*, as well as *srmGIV* and *srmGV*, to give pKOS232-182.

The *chmGI-II* cassette as a NdeI-HindIII fragment (as described in [17]), the *srmGIII-V* cassette as a HindIII-SpeI fragment, and the 8 kb NdeI-SpeI fragment of pKOS232-189 were joined in a three-fragment ligation and recovered by in vitro packaging (Gigapak Gold III, Stratagene). The resulting plasmid, pKOS232-184A, was introduced into *E. coli* DH5α/pUB307 and then into *S. fradiae* K159-1/pKOS244-017A by conjugation.

Analysis of Fermentation Products

Cultures were grown for 7–8 days at 28°C in R media [30]. Culture broth samples were prepared for LC-MS analysis by centrifugation and filtration through 0.2 μm filters or by ethyl acetate extraction, drying, and dissolution of the residue in methanol. Aqueous samples were subjected to on-line solid-phase extraction before switching to the column for fractionation. A Metachem Metasil Basic column (4.6 × 150 mm, 5 μm particle) was used with an 8 min linear gradient from 35% to 100% buffer B (4:1 MeCN:MeOH, 5 mM NH_4Ac) in buffer A (5 mM NH_4Ac in water) at a flow rate of 1 ml/min. There was simultaneous detection by API mass spectrometry (Turbo Ionspray source) and UV absorption at 280 nm.

Isolation and Structural Characterization of Compounds

To isolate 4″-despropionyl-14-methylniddamycin (DPMN), clarified fermentation broth (250 ml) was adjusted to pH 9.5 with 1 N NaOH and extracted with $CHCl_3$ (3 × 300 ml). The organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated in vacuo to provide an amber oil (~1 g). The oil was applied to a silica flash column and eluted with a gradient of 25%–55% acetone/hexane (+ 2% NEt_3). Fractions eluting in 40% acetone/hexane (+2% NEt_3) were pooled and concentrated in vacuo to provide a solid that was dissolved in a minimum amount of CH_2Cl_2 and precipitated with hexane. The solvent was removed in vacuo to provide DPMN (0.20 g, 0.283 mmol) as a white powder. ^1H NMR ($CDCl_3$, 400 MHz) δ ppm 1.06 (d, 3H, J = 6.4 Hz), 1.19 (m, 9H), 1.24 (m, 6H), 1.48 (m, 1H), 1.56 (m, 1H), 1.72 (d, 1H, J = 3.4 Hz), 1.85 (m, 1H), 1.99 (d, 1H, J = 14.4 Hz), 2.22 (m, 2H), 2.40 (m, 2H), 2.44 (s, 6H), 2.50 (m, 1H), 2.70 (m, 2H), 2.90 (m, 1H), 3.09 (d, 1H, J = 10.0 Hz), 3.23 (m, 2H), 3.48 (dd, 1H, J = 7.6, 10.4 Hz), 3.51 (s, 3H), 3.75 (d, 1H, J = 10.0 Hz), 4.02 (m, 2H), 4.37 (s, 1H, J = 7.6, 10.4 Hz), 4.82 (dquart, 1H, J = 10.0, 6.4 Hz), 5.03 (d, 1H, J = 3.4 Hz), 5.85 (dd, 1H, J = 14.8, 10.0 Hz), 6.11 (dd, 1H, J = 14.8, 11.2 Hz), 6.28 (d, 1H, J = 14.8 Hz), 7.19 (dd, 1H, J = 14.8, 11.2 Hz), 9.37 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 15.87, 17.29, 17.88, 18.16, 18.94, 25.31, 31.89, 32.32, 38.03, 40.81, 41.88, 43.21, 44.67, 45.75, 61.81, 65.93, 67.58, 68.70, 69.35, 71.70, 73.05, 74.95, 73.05, 76.33, 79.16, 85.28, 96.39, 103.94, 122.29, 129.41, 143.36, 147.74, 173.22, 202.35, 202.45; HRMS calc. for $C_{35}H_{60}NO_{13}$ $[MH]^+$: 714.40592; found: 714.40808.

To isolate 6-carboxymethyl-6-desethyl-14-methyl-5-O-mycaminosylplatenolide (CDMP), clarified fermentation broth (1 liter) was applied to a column of XAD-16 resin (500 ml). The column was eluted with water (3.5 liter), removing most of the yellow material, and then with 33%, 50%, and 100% MeOH/H_2O. Fractions eluting with 100% MeOH were pooled, and solvent was removed in vacuo to provide a brown oil (~920 mg). The oil was applied to a silica flash column and first eluted with 10%–100% acetone/hexane (+2% NEt_3) to remove nonpolar material, followed by 0%–10% MeOH/CH_2Cl_2 (+2% NEt_3). Fractions eluting in 10% MeOH/CH_2Cl_2 (+2% NEt_3) were pooled and concentrated in vacuo to provide a yellow oil. The material was applied to a second silica flash column, eluting with 0%–20% MeOH/CH_2Cl_2 (+2% NEt_3). Fractions eluting in 5%–10% MeOH/CH_2Cl_2 (+2% NEt_3) were pooled and concentrated in vacuo to provide a yellow solid (~40 mg). This material was subjected to HPLC (150 × 212 mm 5 μ MetaChem Polaris C-18 column, 10 ml/min), eluting with a gradient of 30%–100% A/B (A = 5 mM NH_4OAc CH_3CN:MeOH [4:1]; B = 5 mM NH_4OAc). Fractions eluting in 45% A/B were pooled and concentrated in vacuo. Residual NH_4OAc was removed by application of the material to a silica flash plug and elution with 10% MeOH/CH_2Cl_2 (+2% NEt_3). The product was concentrated in vacuo to provide CDMP (0.018 g, 0.031 mmol) as a white solid. ^1H NMR (CD_3OD, 400 MHz) δ ppm 1.09 (d, 3H, J = 6.4 Hz), 1.18 (d, 3H, J = 7.2 Hz), 1.30 (m, 6H), 1.67 (m, 3H), 2.29 (m, 2H), 2.37 (d, 1H, J = 15.6 Hz), 2.56 (m, 3H), 2.95 (s, 6H), 3.11 (t, 1H, J = 10.0 Hz), 3.25 (d, 1H, J = 10.6 Hz), 3.31 (m, 1H), 3.36 (m, 1H), 3.52 (m, 1H), 3.55 (s, 3H), 3.88 (d, 1H, J = 10.6 Hz), 4.03 (d, 1H, J = 9.6 Hz), 4.52 (d, 1H, J = 7.2 Hz), 4.78 (dquart, 1H, J = 10.0, 6.4 Hz),

5.91 (dd, 1H, J = 14.8, 10.0 Hz), 6.31 (dd, 1H, J = 14.8, 11.2 Hz), 6.57 (d, 1H, J = 14.8 Hz), 7.19 (dd, 1H, J = 14.8, 11.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 14.79, 16.38, 16.42, 16.87, 41.88, 32.06, 33.64, 34.83, 38.29, 40.78, 44.58, 45.16, 60.89, 67.80, 68.68, 69.61, 70.91, 72.67, 72.74, 79.33, 85.66, 102.99, 122.75, 129.76, 143.58, 147.78, 171.99, 178.24, 204.49; HRMS calc. for $C_{29}H_{49}NO_{11}$ $[MH]^+$: 586.32219; found: 586.32024.

To purify 12,16-didesmethyl-DMT, clarified fermentation broth (1.15 liter) was adjusted to pH 7.8 with $NaHCO_3$, the solution filtered, and the filtrate extracted with CH_2Cl_2 (4 × 850 ml). The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo to provide a yellow semisolid (377 mg). This material was applied to a silica flash column, eluting with 10%–60% acetone/hexane (+2% NEt_3). Fractions eluting in 30% acetone/hexane (+2% NEt_3) were pooled and solvent was removed in vacuo to provide an off-white solid (73 mg). This material was subjected to HPLC (150 × 212 mm 5 μ MetaChem Polaris C-18 column, 10 ml/min), eluting with a gradient of 50%–100% A/B (A = 5 mM NH_4OAc CH_3CN:MeOH [4:1]; B = 5 mM NH_4OAc). Fractions eluting between 20 and 25 min were pooled and partitioned between CH_2Cl_2 and aqueous saturated $NaHCO_3$ to remove residual NH_4OAc. The organic extracts were combined, dried over Na_2SO_4, and filtered. Hexane was added until the solution became cloudy and solvent was removed in vacuo to provide a white solid (25.5 mg). The material was subjected to a second round of HPLC, as just described, to provide 12,16-didesmethyl-DMT (~12 mg) as a white powder. The structure was verified by ^{1}H, ^{13}C, COSY, multiplicity edited HSQC, and HMBC. ^{1}H NMR ($CDCl_3$, 400 MHz), δ ppm 9.65 (s, 1H, 19-CHO), 7.26 (dd, 1H, 11-H, $J_{11\text{-}10}$ = 14.8 Hz, $J_{11\text{-}12}$ = 14.8 Hz), 6.28 (d, 1H, 10-H, $J_{10\text{-}11}$ = 15.2 Hz), 6.18 (dd, 1H, 12-H), 6.15 (dd, 1H, 13-H), 5.10 (dq, 1H, 15-H, $J_{15\text{-}14}$ = 10 Hz, $J_{15\text{-}16}$ = 6.4 Hz), 5.00 (d, 1H, 1″-H, $J_{1''\text{-}2''}$ = 3.2 Hz), 4.20 (d, 1H, 1′-H, $J_{1'\text{-}2'}$ = 7.2 Hz), 4.05 (dq, 1H, 5″-H, J = 9.6 Hz & 6 Hz), 3.84 (d, 1H, 3-H, $J_{3\text{-}2}$ = 10 Hz), 3.74 (d, 2H, 21-H, $J_{21\text{-}14}$ = 4.8 Hz), 3.70 (d, 1H, 5-H, $J_{5\text{-}4}$ = 9.2 Hz), 3.52 (dd, 1H, 2′-H, $J_{2'\text{-}1'}$ = 7.6 Hz, $J_{2'\text{-}3'}$ = 10.2 Hz), 3.24 (1H, 4′-H, overlapped with 5′), 3.24 (1H, 5′-H, overlapped with 4′), 2.93 (d, 1H, 4″-H, $J_{4''\text{-}5''}$ = 10 Hz), 2.87 (dd, 1H, 18a-H, $J_{18a\text{-}18b}$ = 18 Hz, $J_{18\text{-}19}$ = 8.4 Hz), 2.56 (m, 1H, 8-H), 2.56 (1H, 2a-H, overlapped with 8-H), 2.47 (s, 6H, 3′-NMe_2), 2.46 (1H, H-3′, overlapped with 3′-NMe_2), 2.35 (m, 1H, 14-H), 2.04 (dd, 1H, 2″a-H), 2.00 (dd, 1H, 2b-H), 2.00 (dd, 1H, 2″-a), 1.74 (dd, 1H, 2″b-H, $J_{2''a\text{-}2''b}$ = 14.4, Hz $J_{1''\text{-}2''}$ = 3.6 Hz), 1.68 (m, 1H, 4-H), 1.59 (m, 1H, 6-H), 1.32 (d, 3H, 16-H, $J_{16\text{-}15}$ = 6.4 Hz), 1.27 (d, 3H, 6″-H, $J_{6''\text{-}5''}$ = 6.4 Hz), 1.24 (m, 2H, 7-H), 1.23 (d, 3H, 6′-H, $J_{6'\text{-}5'}$ = 7.6 Hz), 1.22 (s, 3H, 3″-Me), 1.17 (d, 3H, 20-H, $J_{20\text{-}8}$ = 6.8 Hz), 1.00 (d, 3H, 17-H, $J_{17\text{-}4}$ = 6.8 Hz) . ^{13}C NMR ($CDCl_3$, 100 MHz), δ ppm 203.0 (C-9), 202.7 (C-19), 173.4 (C-1), 143.5 (C-13), 143.1 (C-11), 131.8 (C-12), 122.6 (C-10), 103.6 (C-1′), 96.4 (C-1″), 80.8 (C-5), 76.3 (C-4″), 74.9 (C-4′), 73.1 (C-5′), 71.7 (C-2′), 69.5 (C-15), 69.4 (C-3″), 69.4 (C-3), 68.6 (C-3′), 65.9 (C-5″), 61.8 (C-21), 53.5 (C14), 44.6 (C-8), 43.4 (C-18), 41.9 (3′-NMe_2), 40.8 (C-2″), 39.8 (C-2), 32.1 (C-4), 31.8 (C-6), 29.6 (C-7), 25.3 (C-3″-Me), 18.9 (C-6′), 18.3 (C-16), 18.2 (C-6″), 17.3 (C-20), 9.0 (C-17). HRMS calc. for $C_{36}H_{60}NO_{13}$ $[MH]^+$: 714.40592, found: 714.40457.

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Engineered biosynthesis of 16-membered macrolides that require methoxymalonyl-ACP precursors in *Streptomyces fradiae*

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Abstract Development of host microorganisms for heterologous expression of polyketide synthases (PKS) that possess the intrinsic capacity to overproduce polyketides with a broad spectrum of precursors supports the current demand for new tools to create novel chemical structures by combinatorial engineering of modular and other classes of PKS. *Streptomyces fradiae* is an ideal host for development of generic polyketide-overproducing strains because it contains three of the most common precursors —malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA—used by modular PKS, and is a host that is amenable to genetic manipulation. We have expanded the utility of an overproducing *S. fradiae* strain for engineered biosynthesis of polyketides by engineering a biosynthetic pathway for methoxymalonyl-ACP, a fourth precursor used by many 16-membered macrolide PKS. This was achieved by introducing a set of five genes, *fkbG–K* from *Streptomyces hygroscopicus*, putatively encoding the methoxymalonyl-ACP biosynthetic pathway, into the *S. fradiae* chromosome. Heterologous expression of the midecamycin PKS genes in this strain resulted in 1 g/l production of a midecamycin analog. These results confirm the ability to engineer unusual precursor pathways to support high levels of polyketide production, and validate the use of *S. fradiae* for overproduction of 16-membered macrolides derived from heterologous PKS that require a broad range of precursors.

Introduction

Polyketides represent an extremely diverse class of metabolites that include important therapeutic agents for antibacterial (erythromycin, tylosin, rifamycin), immunosuppressive (FK506, rapamycin), cholesterol-lowering (lovastatin), and other uses (O'Hagan 1991). An understanding of the genetic and biochemical principals underlying polyketide biosynthesis has led to the development of technologies for creating novel chemical structures by the directed and combinatorial engineering of modular and other classes of polyketide synthases (PKS) (Rodriguez and McDaniel 2001). However, engineering of PKS genes in the chromosome of organisms that typically produce polyketides (e.g., actinomycetes) is often a difficult and tedious, if not impossible, process. Consequently, several host-vector systems for heterologous PKS expression have been developed for rapid engineering of novel polyketides (Hu et al. 1999; Kealey et al. 1998; McDaniel et al. 1993; Pfeifer et al. 2001; Tang et al. 2000; Xue et al. 1999; Zhao et al. 1999; Ziermann and Betlach 1999). In general, genetically modified PKS can be more easily constructed in *Escherichia coli* cloning strains and introduced into a host capable of polyketide production. Two potential problems with this approach, however, are low production titers of the compound of interest in the heterologous host compared to the naturally producing organism, and the absence of uncommon or unusual precursor metabolites necessary for biosynthesis of the desired polyketide. In this work, we have addressed both obstacles for production of the 16-membered macrolide class of polyketides, which includes important antibiotics such as tylosin, spiramycin, and midecamycin.

Several PKS gene clusters, which provide potential precursor metabolite genes that may be used for assembly of different polyketide building blocks in organisms that normally lack them, have now been sequenced. For example, polyketide gene clusters containing a set of genes for production of methoxymalonyl-ACP precursor have been described (Carroll et al. 2002; Wu et al. 2000). Recently, it was shown that heterologous expression of a set of five genes, *asm13–17*, from *Actinosynnema pretiosum* was sufficient for the formation of the substrate α-methoxymalonate polyketide chain extension unit in *Streptomyces lividans*, leading to production of 2-

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desmethyl-2-methoxy-6-deoxyerythrolide B by expression of an engineered 6-deoxyerythronolide B synthase (DEBS) gene (Kato et al. 2002). Clearly, it would be advantageous to have host microorganisms that possess the intrinsic capacity to overproduce polyketides with a broad spectrum of precursors.

We recently examined overproduction characteristics of an industrial *Streptomyces fradiae* strain and an industrial *Saccharopolyspora erythraea* strain (Rodriguez et al. 2003) that had been optimized for production of tylosin and erythromycin, respectively. The potential of such hosts for engineered polyketide overproduction was demonstrated by the enhanced production of erythromycin analogs from genetically engineered erythromycin PKSs compared to production in a non-optimized host. Furthermore, a high production level of tylosin was achieved with the *S. fradiae* overproducer strain when the *tylG* PKS from the industrial host was replaced by a *tylG* PKS from a low producer strain, suggesting that this host could be used for overproduction of novel 16-membered macrolides from heterologous and engineered PKS (Rodriguez et al. 2003).

Biosynthesis of tylosin requires the precursors malonyl-CoA, methylmalonyl-CoA, and the less common ethylmalonyl-CoA. Many 16-membered macrolides, including midecamycin and spiramycin, also require the unusual precursor methoxymalonyl-ACP (Fig. 1) (Baltz et al. 1983). Here we extend the overproducing capabilities of the *S. fradiae* strain by introducing the methoxymalonyl-ACP biosynthetic pathway of FK520 (ascomycin) from *Streptomyces hygroscopicus* (Wu et al. 2000). The resulting strain is capable of producing the four different precursors malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA and methoxymalonyl-ACP, and produced more than 1 g/l of a 16-membered macrolide through heterologous expression of the midecamycin (*mdm*) PKS.

Materials and methods

Strains, culture conditions, and genetic manipulation

S. fradiae K159-1, which contains a deletion of the *tylGI-V* genes (Rodriguez et al. 2003), was propagated on AS-1 agar medium (Baltz 1980) and transformed by conjugation using *E. coli* DH5α/pUB307 containing the donor plasmid. Conjugation experiments were performed according to Rodriguez et al. (2003). Transconjugants were selected with 3 ml soft agar containing 1 mg nalidixic acid and either 1 mg apramycin or 2.5 mg kanamycin, as required. Transformants were further propagated on AS-1 agar medium containing 30 mg/l apramycin or 50 mg/l kanamycin. DNA cloning and manipulation were performed in *E. coli* DH5α.

Construction of ACP methoxymalonyl-CoA pathway expression vector

A kanamycin resistance version of pSET152 (Bierman et al. 1992) was made by cloning a *neo* cassette from plasmid pFDneoS (Denis and Brzezinski 1991) digested with *Sac*I-*Xba*I, into pSET152 digested with *Sac*I-*Nhe*I, resulting in plasmid pKOS244-4. The pSAM2 *int-attP* genes were cloned in pKOS244-4 under the control of the *int*(ΦC31) promoter. A *Bam*HI fragment containing the *int-attP* (pSAM2) from pKOS38-67 (Xue et al. 1999) was cloned in pKOS244-4 digested with *Bam*HI-*Bcl*I to make pKOS244-11. The *fkbG-K* genes from the biosynthetic gene cluster of FK520 (ascomycin) in *S. hygroscopicus* (Wu et al. 2000) were cloned in the above vector under the control of the *tylGIp* promoter to give pKOS244-17a as follows. The *fkbG* gene was amplified by PCR using forward primer, 5′-AAGAATTCATATGGCTAATCAGATAACCCTG-3′, and reverse primer 5′-TTGAGCTCTCACCGTTTCCGCAGCAGGGTG-3′ (underlined restriction sites *Eco*RI, *Nde*I and *Sac*I were used for cloning) from pKOS034-120. The *fkbH-K* operon was isolated as a 4.2 kb *Sac*I-*Xho*I fragment (*Xho*I end-filled to make it

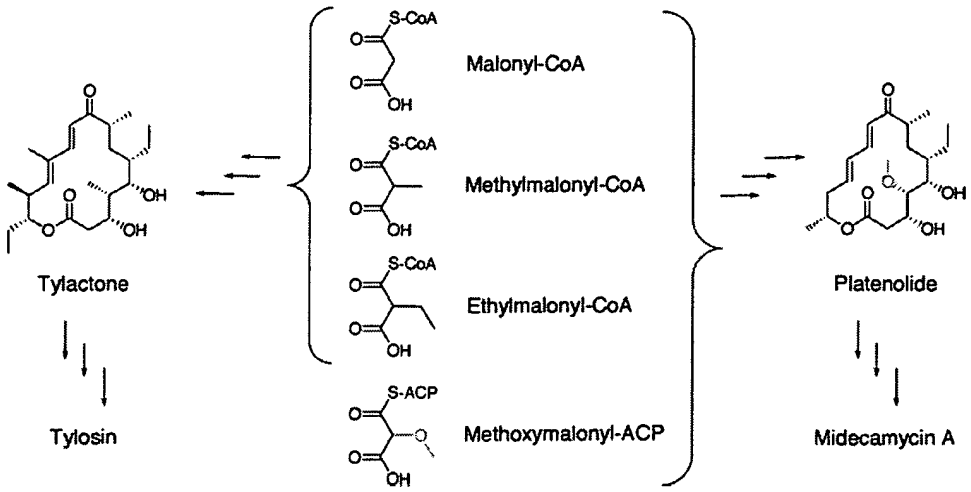

Fig. 1 Precursors required for tylactone and platinolide biosynthesis. Tylactone, the product of the tylosin polyketide synthase (PKS), is synthesized from two malonyl-CoA, four methylmalonyl-CoA, and one ethylmalonyl-CoA precursor. Platinolide, the product of the midecamycin PKS, requires a methoxymalonate unit instead of a methylmalonate unit in the penultimate condensation step. Tylactone and platenolide are subsequently converted to tylosin and midecamycin by post-PKS glycosylation, acylation and oxidation reactions

blunt) from pKOS034-120 and cloned into pIJ2925 digested with *Sac*I-*Sma*I (Janssen and Bibb 1993). The *fkbG* PCR product was verified by sequencing, and cloned upstream of the *fkbH-K* operon as an *Eco*RI-*Sac*I fragment to create plasmid pKOS210-12b. The *fkbG-K* operon was cloned as an *Nde*I-*Bgl*II fragment in pKOS159-3 under the control of the *tylGlp* promoter (Rodriguez et al. 2003), and the entire methoxymalonate expression cassette was moved to pKOS244-11 as an *Eco*RI fragment to create pKOS244-17a (Fig. 2b). All plasmids used are listed in Table 1.

Construction of the *mdm* PKS expression vector

The *mdm* PKS expression plasmid, pKOS244-20, was constructed similarly to the *tyl* PKS expression plasmid pKOS244-5 described previously (Rodriguez et al. 2003). The *mdm* PKS genes from *Streptomyces mycarofaciens* were obtained from two cosmids, pCOM1 and pCOM2, provided by Meiji Seika Kaisha, Tokyo, Japan (unpublished data). The *mdm* PKS was pieced together to give the expression vector pKOS244-20 as follows. PCR was used to obtain a 410 bp fragment of the 3′ end of the *mdmGV* gene from pCOM1 using the oligonucleotides: 5′-CCGAATTCGATCGATGTGTACACGCTGCAGCACG-GATCCCGGCGCGATGGGCG-3′ and 5′-CGTCTAGAT-CAGTGTTTTCCTCCGGTGA-3′. The PCR product was digested with *Xba*I and *Eco*RI and ligated to a similarly cut pLITMUS28 to generate the plasmid pKOS231-26. PCR was also used to generate a 136 bp fragment of the 5′ end of *mdmGI* using pCOM2 as the template with the following oligonucleotides: 5′-GCGATGCATCCTTAAT-TAAGGAGGACAGGAATGCTGGTGTCTGGA-GATCTCGT-3′ and 5′-CGTCTAGACAGAATTCTTC-GACTCCCG-3′. This PCR product was cut with *Nsi*I and *Xba*I and ligated with pLITMUS28 cut with the same enzymes to create pKOS231-33.

pCOM 1 was digested with *Bam*HI and *Pst*I and the ~1.9 kb fragment was ligated into pKOS231-26 cut with the same enzymes to create pKOS231-28. The ~2.3 kb insert from this plasmid was then moved by cutting both pKOS231-28 and pKOS231-33 with *Afl*II and *Eco*RI and then ligating the pieces to make pKOS231-35. pKOS231-35 was then cut with *Bsr*GI and *Pst*I and ligated to a ~12 kb fragment from pCOM2 cut with the same enzymes to generate the plasmid pKOS231-36. The genes in this plasmid were then cut with *Avr*II and *Nsi*I and the ~14 kb

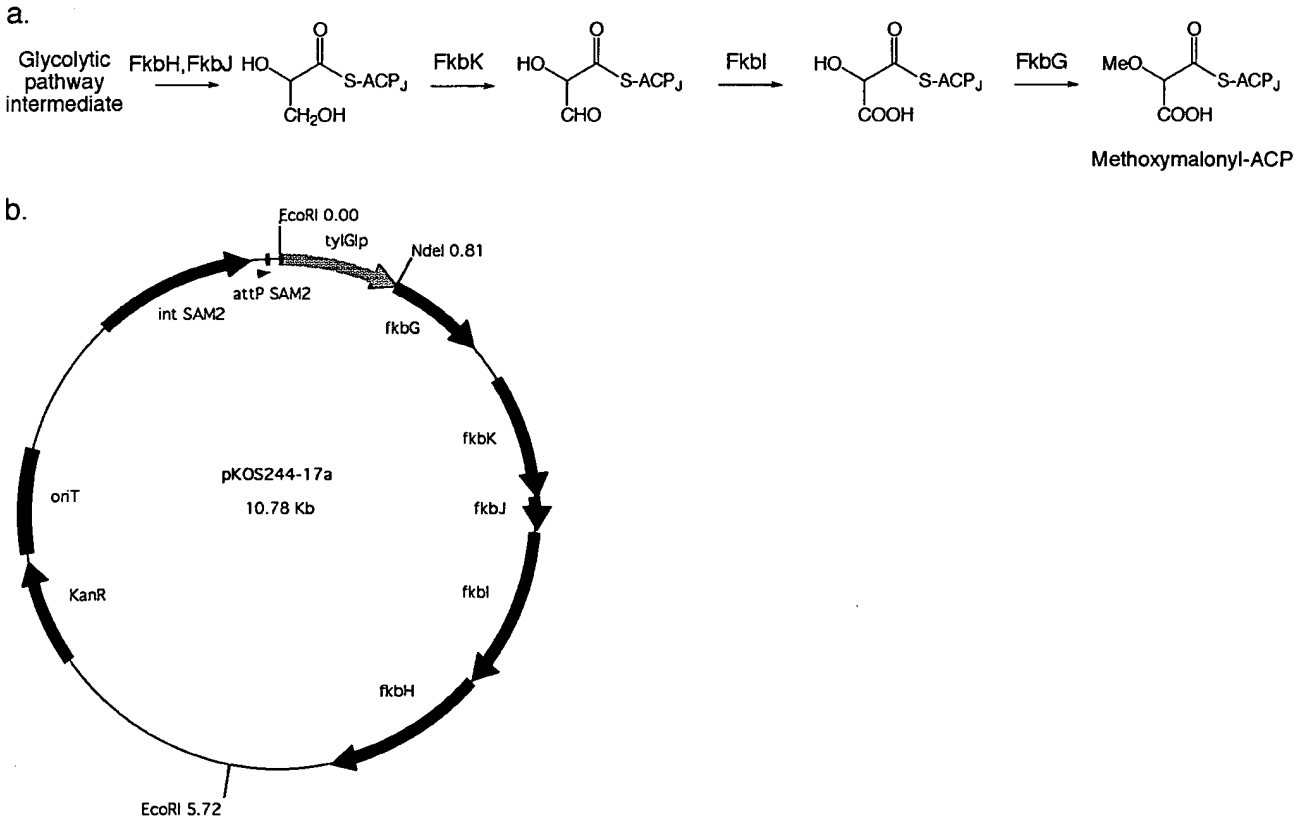

Fig. 2 **a** Proposed biosynthetic route for production of methoxymalonyl-ACP in *Streptomyces hygroscopicus* ATCC 14891 (Wu et al. 2000). FkbJ (ACPj) is homologous to ACP. Although the function of FkbH is unknown, it is proposed to be involved in the formation of glyceryl-ACPJ from a three-carbon intermediate of the glycolytic pathway. FkbK is homologous to enzymes that oxidize 3-hydroxyacyl-CoA compounds to 3-oxoacyl-CoA compounds, consistent with the reaction shown. FkbI is homologous to enzymes that oxidize saturated acyl-CoA compounds to 2,3-enoyl-CoA compounds using FAD cofactor. FkbG is homologous to *O*-methyltransferase. **b** Map of plasmid pKOS244-17a for expression of the corresponding genes

Table 1 List of all plasmids used

Plasmids	Description	Reference
pFDneoS	Contains neomycin/kanamycin resistance gene	Denis and Brzezinski (1991)
pIJ2925	pUC18 derivative	Janssen and Bibb (1993)
pKOS34-120	Vector with *fkbG-K* genes	Wu et al. (2000)
pKOS38-67	Vector with *int-attP* genes from pSAM2 vector	Xue et al. (1999)
pKOS159-3	pCRscript with *tylGIp* promoter	Rodriguez et al. (2003)
pKOS159-31	pSET152 with *eryAI-III* genes under *eryAIp* promoter	Rodriguez et al. (2003)
pLITMUS28	Cloning vector	New England Biolabs, Beverly, Mass.
pLITMUS29	Cloning vector	New England Biolabs
pLITMUS38	Cloning vector	New England Biolabs
pSET152	Contains an *oriT* for conjugal transfer, the *int* (ΦC31) gene and apramycin resistance gene	Bierman et al. (1992)
pUB307	Helper element to mobilize plasmid from *Escherichia coli* to *Streptomyces* spp.	Piffaretti et al. (1988)
pCOM1	Cosmid containing *mdm* PKS genes	Meiji Seika Kaisha, Tokyo, Japan
pCOM2	Cosmid containing *mdm* PKS genes	Meiji Seika Kaisha
pKOS210-12b	pIJ2925 with *fkbG-K* genes as an operon	This work
pKOS231-26	pLITMUS28 with PCR product of *mdm* PKS genes	This work
pKOS231-28	pKOS231-26 with a 1.9 kb *Bam*HI-*Pst*I fragment from pCOM1	This work
pKOS231-33	pLITMUS28 with PCR product of *mdm* PKS genes	This work
pKOS231-35	pKOS231-33 with a 2.3 kb *Afl*II-*Eco*RI fragment from pKOS231-28	This work
pKOS231-36	pKOS231-35 with a 12 kb *Bsr*GI-*Pst*I fragment from pCOM2	This work
pKOS231-45	pLITMUS29 with a 14 kb *Avr*II-*Nsi*I fragment from pKOS231-36	This work
pKOS231-41	pLITMUS38 with a 16 kb *Mfe*I-*Sca*I fragment from pCOM2 and a 9.9 kb *Bsr*GI-*Sca*I fragment from pCOM1	This work
pKOS231-46	pKOS231-45 with a 23 kb *Mfe*I-*Bsr*GI fragment from pKOS231-41	This work
pKOS244-4	pSET152 with neomycin/kanamycin resistance gene	This work
pKOS244-11	pKOS244-4 derivative where *int* (ΦC31) gene was replaced by *int-attP* genes of pSAM2	This work
pKOS244-17a	pKOS244-11 with *fkbG-K* genes under *tylGIp* promoter	This work
pKOS244-18	pKOS159-3 with 5′ end of *mdm* PKS gene	This work
pKOS244-20	pSET152 derivative containing *mdm* PKS genes under *tylGIp* promoter	This work

fragment was ligated to pLITMUS29 cut with the same enzymes to create plasmid pKOS231-45.

The genes *mdmGI–GII* were isolated by digesting pCOM2 with *Mfe*I and *Sca*I to obtain a ~16 kb fragment and pCOM1 with *Bsr*GI and *Sca*I to obtain a ~9.9 kb fragment. These two fragments were ligated to *Mfe*I/*Bsr*GI-digested pLITMUS38 to produce the plasmid pKOS231-41. To bring *mdmGI–GV* together, pKOS231-41 was digested with *Mfe*I and *Bsr*GI to isolate the ~23 kb fragment. This fragment was then ligated to pKOS231-45 also cut with *Mfe*I and *Bsr*GI to create pKOS231-46. To create pKOS244-20, an *Nde*I-*Mfe*I linker containing the 5′ end of *mdmGI* was cloned downstream of the *tylGIp* promoter to give pKOS244-18 using two annealed oligonucleotides (forward 5′-TATGCTGGTGTCTGGAGATCTCGTGACTTCCCGAATTGACGACCGATCCGATGCAATTGGAGCT′; reverse 5′-CCAATTGCATCGGATCGGTCGTCAATTCGGGAAGTCACGAGATCTCCAGACACCAGCA′). A *Mfe*I-*Spe*I fragment containing *mdm* PKS genes from pKOS231-46 and a *Mfe*I-*Pst*I fragment from pKOS244-18 were then inserted into the *Nsi*I-*Spe*I sites of pKOS159-31 (Rodriguez et al. 2003) to yield the final expression construct pKOS244-20.

Fermentation conditions for *S. fradiae* strains

S. fradiae strains were analyzed in 50 ml RM medium (per liter: wheat flour, 15 g; corn gluten enzymatic hydrolysate, 10 g; beet molasses, 25 g; brewer's yeast, 2.5 g; $(NH_4)_2HPO_4$, 1 g; NaCl, 1 g; $CaCO_3$, 2 g and 1.7 ml soybean oil per 50 ml). Seed cultures were grown in 5 ml TSB medium (containing 30 mg/l apramycin) at 30°C for 48 h and used to inoculate 50 ml RM medium (30 mg/l apramycin) in a 250 ml baffled Erlenmeyer flask. After 7 days growth at 30°C, the culture broth was analyzed for polyketide production by HPLC (Metachem Metasil Basic column, 4.6×150 mm, 5 μm particle; MetaChem, Lake

Forest, Calif.) using a linear gradient from 15 to 100% organic phase (56% methanol, 5 mM ammonium acetate) at 1 ml/min over 7 min. Compounds were identified by MS and UV absorption at 282 nm. Tylosin standard (Sigma, St. Louis, Mo.; T-6134) was used to generate a calibration curve for titer determination.

Isolation and characterization of 3,4″-didespropionylmidecamycin A3

Culture broth (1 l) was spun down in a centrifuge (8,000 *g*). The pH of the supernatant was adjusted to >9 with NH_4OH. The broth was extracted with ethyl acetate (3×4 l) and the combined organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. The compound was purified on a silica gel column (acetone/hexane=5–50% with 1% triethylamine) to obtain 200 mg crude product. The compound was further purified by HPLC using a reverse phase column (Metachem Metasil Basic column, 21×150 mm, 5 μm particle, mobile phase from 20% solvent B to 75% solvent B over 30 min, solvent A=5 mM NH_4OAc in H_2O, solvent B=acetonitrile/methanol=4/1 with 5 mM NH_4OAc). MS ($M + H^+$)=701, the structure of the product was characterized by ^1H, ^{13}C, COSY, multiplicity-edited HSQC, and HMBC. ^1H NMR ($CDCl_3$, 400 MHz), δ (ppm), 9.66 (1H, 18-CHO), 7.26 (1H, 11-H), 6.32 (1H, 10-H), 6.14 (2H, 12-H), 6.14 (1H, 13-H), 5.22 (1H, 15-H), 5.06 (1H, 1″-H), 4.40 (1H, 1′-H), 4.07 (1H, 5″-H), 4.05 (1H, 5-H), 3.78 (1H, 3-H), 3.54 (3H, 4-OMe), 3.50 (1H, 2′-H), 3.26 (1H, 4′-H), 3.25 (1H, 5′-H), 3.12 (1H, 4H), 2.93 (1H, 4″-H), 2.76 (1H, 2-Ha), 2.72 (1H, 17-Ha), 2.55 (1H, 8-H), 2.51 (1H, 14-Ha), 2.50 (6H, 3′-NMe_2), 2.45 (1H, 3′-H), 2.45 (1H, 17Hb), 2.25 (1H, 2-Hb), 2.18 (1H, 14-Hb), 2.04 (1H, 2″-Ha), 1.88 (1H, 6-H), 1.75 (1H, 2″-Hb), 1.61 (1H, 7-Ha), 1.51 (1H, 7-Hb), 1.32 (3H, 16-H_3), 1.29 (3H, 6″-H_3), 1.23 (3H, 6′-H_3), 1.22 (3H, 3″-Me), 1.20 (3H, 19-H_3). ^{13}C NMR ($CDCl_3$, 100 MHz), δ (ppm), 202.5 (C-18), 202.4 (C-9), 173.5 (C-1), 143.3 (C-11), 141.2 (C-13), 131.7 (C-12), 122.3 (C-10), 104.0 (C-1′), 96.5 (C-1″), 85.4 (C-4), 79.3 (C-5), 76.4 (C-4″), 75.0 (C-4′), 73.1 (C-5′), 71.8 (C-2′), 69.4 (C-3″), 68.9 (C-15), 68.8 (C-3′), 67.7 (C-3), 66.0 (C-5″), 61.9 (4-OMe), 44.8 (C-8), 43.3 (C-17), 42.0 (3′-NMe_2), 41.6 (C-14), 40.9 (C-2″), 38.0 (C-2), 32.4 (C-7), 32.0 (C-6), 25.4 (3″-Me), 20.3 (C-16), 19.0 (C-6′), 18.2 (C-6″), 17.4 (C-19).

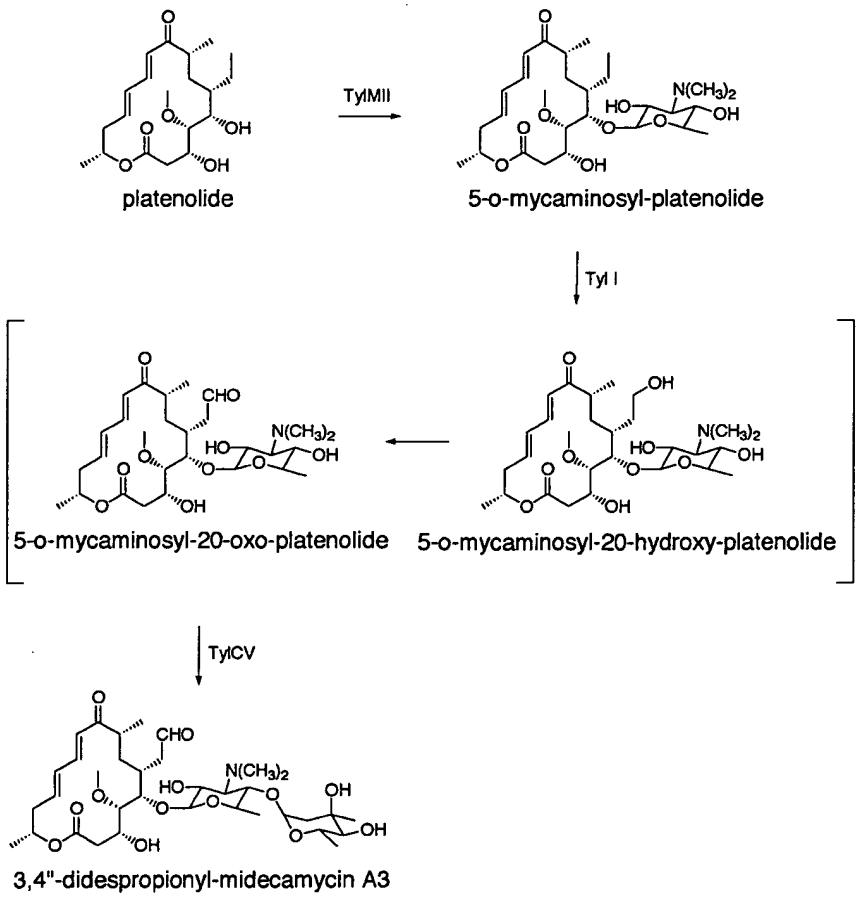

Fig. 3 Proposed pathway for biosynthesis of a 16-membered macrolide in *Streptomyces fradiae*. Post-PKS steps involve glycosylation by TylMII, which adds mycaminose to the 5-hydroxyl group, hydroxylation at C-20 by the cytochrome P450 TylI, and attachment of mycarose by TylCV

Results

Construction of the methoxymalonyl-ACP pathway in *S. fradiae*

Recently we reported a PKS gene delivery (up to 50 kb) and expression system for a tylosin-overproducing strain of *S. fradiae* (Rodriguez et al. 2003). In this system, PKS genes targeted for expression are cloned into a vector based on the ΦC31 site-specific recombination phage and delivered by conjugal transfer from *E. coli*. The host used for expression, *S. fradiae* K159-1, is an engineered derivative of the overproducer in which the tylosin PKS genes, *tylGI–V*, were deleted. In order to introduce the methoxymalonyl-ACP pathway, a set of five genes, *fkbG–K* from the FK520 gene cluster of *S. hygroscopicus*, was assembled in plasmid pKOS244-17a (Fig. 2b), a site-specific integrating vector modified from the pSAM2 integration system (Xue et al. 1999). pKOS244-17a contains the neomycin resistance gene, *neoD*, for selection, and the integrase gene, *int*, and attachment site, *attP*, from pSAM2. The *int* gene is under the control of the ΦC31 *int* promoter rather than its natural promoter. Typically ΦC31 derivative vectors integrate with greater efficiency than pSAM-based vectors in most actinomycetes, and here pKOS244-17a was found to integrate with an efficiency similar to that of pSET152 (ΦC31 derivative). The pKOS244-17a plasmid is also compatible with ΦC31-based vectors, permitting integration of PKS genes and precursor pathway genes on separate plasmids. The *fkbG–K* cluster was cloned under the control of the *tylGIp* promoter in pKOS244-17a and the plasmid was introduced into *S. fradiae* K159-1 by conjugal transfer from *E. coli*. Functional expression of the *fkbG–K* genes was confirmed by co-expression with the *mdm* PKS genes as described below.

Cloning and expression of *mdm* PKS in *S. fradiae*

The organization and architecture of the midecamycin PKS gene cluster isolated from Meiji's *S. mycarofaciens* strain is analogous to other 16-membered macrolide clusters that have been characterized (i.e., tylosin, niddamycin, spiromycin) (personal communication, Meiji Ltd, Japan). A full *mdm* PKS expression plasmid (pKOS244-20) using *tylGIp* was constructed in a similar way as the tylosin PKS expression plasmid (pKOS244-5) described previously and used to produce >1 g/l tylosin in *S. fradiae* K159-1 (Rodriguez et al. 2003). The expression plasmid pKOS244-20 was transferred to the *S. fradiae* K159-1/pKOS244-17a strain described above by conjugation. Apramycin-resistant transconjugants were isolated and fermented in RM medium. The culture broths of *S. fradiae* K159-1/pKOS244-17a/pKOS244-20 were shown to contain compounds with antibacterial activity based on agar plate bioassays with *Micrococcus luteus* as a tester strain. The culture broths were also subjected to online-extraction LC-MS analysis. A major peak was observed with absorbance at 282 nm with *m/z* 701 [M + H^+] and *m/z* 733 [M + H^+], consistent with the calculated molecular weights for 3,4″-didespropionylmidecamycin A3 (Fig. 3) and its methanol adduct, respectively. Several minor peaks corresponding to other putative intermediates in the proposed biosynthetic pathway shown in Fig. 3 were also detected.

The 3,4″-didespropionylmidecamycin A3 compound was isolated and characterized by NMR spectroscopy. The ^1H chemical shift at 3.49 ppm established the presence of a methoxy carbon and is consistent with that of midecamycin. The *S. fradiae* K159-1 host transformed alone with pKOS244-20 cultures did not produce any of the above compounds, establishing unequivocally that the methoxymalonate precursor in 3,4″-didespropionylmidecamycin A3 is derived from expression of the *fkbG–K* genes. The production level of this compound was estimated to be 1 g/l.

Discussion

S. fradiae is an ideal host for development of generic polyketide-overproducing strains because it contains three of the four most common precursors found in modular polyketide biosynthesis—malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA—and is a host that is amenable to genetic manipulation (Baltz and Seno 1988). We have broadened the utility of this host for engineered biosynthesis of polyketides by engineering a fourth precursor pathway, methoxymalonyl-ACP, at levels sufficient to obtain at least 1 g/l of a midecamycin analog. To introduce the genes for the methoxymalonyl-ACP pathway, a new integrating vector combining elements of pSAM2 and ΦC31-based integrating vectors was developed. This new vector is compatible with ΦC31 integrating plasmids and integrates with high efficiency.

This host/vector system has also been used recently for production of additional 16-member macrolides from other heterologous and hybrid PKS (S. Ward, manuscript in preparation). These results reaffirm our previous discovery that the principal cause of overproduction is not due to mutations in the native PKS genes of *S. fradiae* (Rodriguez et al. 2003) and can be used for overproduction of polyketides from heterologous PKS. The design of the *S. fradiae* clean host, where the genes *tylGI–V* were deleted, leaves expression of the other tylosin post-PKS modification pathways intact, including those encoding the biosynthesis and attachment of the three deoxysugars (Rodriguez et al. 2003). From our results it is interesting to note that all post-PKS modifications from tylosin biosynthesis occurred on the platenolide backbone, indicating significant substrate flexibility of the tylosin-modifying enzymes pathway. The modifications include glycosylation by TylMII, which adds mycaminose to the 5-hydroxyl group (Gandecha et al. 1997), oxidation at C-20 by the cytochrome P450 TylI (Merson-Davies and Cundliffe 1994), and attachment of mycarose by TylCV (Bate et al. 2000). This suggests that the *S. fradiae* strain

developed here could be useful for engineering a number of novel 16-membered macrolides in high yield with potentially improved antibacterial properties.

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